# HOST-PARASITE ECOPHYSIOLOGY OF OVERWINTERING

By

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Abstract

To survive extreme winters, parasites must overwinter either in a host, as free-living larvae, or be reintroduced yearly through migratory hosts. This thesis examines interrelations between host-parasite overwintering physiology and behavior in Alaska between the trematode *Ribeiroia ondatrae* and their host, wood frogs *(Lithobates sylvaticus)*. The first chapter examines overwintering physiology and behavior of wood frogs in the field. The second chapter creates a laboratory method for determining physiological responses of wood frogs to environmental transitions from summer to fall. The third chapter examines if and how *R. ondatrae* survive within a frozen wood frog.

Free-living wood frogs investigated over two winters in Fairbanks, AK remained frozen for up to 7 months and survived temperatures as low as -18°C, values much more extreme than those previously reported (Chapter 1). Alaskan wood frogs also synthesized and released approximately one order of magnitude greater concentrations of cryoprotectant (glucose) in multiple tissues than levels previously reported. Wood frogs in the field did not experience the same slow and continuous cooling that researchers routinely subject frogs to under experimental conditions. Instead they cooled at rates of up to -1.5°C $h^{-1}$ for short periods in a diurnal freeze-thaw pattern repeated over one to three weeks until remaining frozen for the rest of winter. Since wood frogs only produce glucose at the initiation of freezing, I hypothesized that freeze-thaw cycling within hibernacula allowed for incremental increases of glucose resulting in higher concentrations in field wood frogs than found in laboratory frozen wood frogs. I compared patterns of diurnal freeze-thaw cycling with the standard laboratory freezing protocols for wood frogs. Wood frogs that experienced multiple freeze-thaw events responded with significant increases in glucose concentration in liver, leg, and heart tissues at each freezing with no significant losses in glucose with each following thaw period (Chapter 2). This incremental increase in glucose within wood frogs may also assist in parasite

survival. Trematode metacercariae may be absorbing host glucose and using this cryoprotectant to enhance their survival (Chapter 3). This result provides evidence that host physiology in winter may both hinder (through freezing) and facilitate (through cryoprotectant production) parasite survival.

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## General Introduction

This thesis is an amalgamation of research traditions of ecophysiology, herpetology, and parasitology in Alaska. Ecophysiology has a long, storied history at the University of Alaska Fairbanks (UAF) stemming from the early works of Drs. Laurence Irving and Per Scholander and their research at the Arctic Research Laboratory in Point Barrow. Irving and Scholander did some of the first research on overwintering ectotherms in the Arctic (Scholander et al., 1953). Laurence Irving went on to be the founding director of the Institute of Arctic Biology at the University of Alaska. For their contributions to Arctic research and ecophysiology the Laurence Irving–Per F. Scholander Memorial Lecture is held in their names (Elsner, 2000).

Research on wood frogs (*Lithobates sylvaticus)* in Alaska also started in the late 1940's with Rodgers Dean Hamilton of John Hopkins University coming to the Arctic to study the distribution of wood frogs and how they survive winter ("Frog hunter tours Arctic", 1948). Unfortunately, Hamilton died in a plane crash in May 1950 before he could publish any of his findings (Maclure, 1950). This tradition of studying wood frogs in Alaska continued at UAF with Drs. Brina Kessel, Clyde Herreid II, and Stephen Kinney in the 1960 who all carried out work on wood frog reproductive and developmental ecology at Ballaine Lake (Kessel, 1965; Herreid and Kinney, 1966; Herreid and Kinney, 1967). Freeze-tolerance in wood frogs was even almost uncovered in a Master's thesis by Michael Kirton in 1974, when he tracked wood frogs to winter hibernacula and recorded hibernacula temperature (Kirton, 1974).

Parasitology has also defined research in Alaska through Dr. Robert Rausch, a pioneer in Alaskan parasitology. He began his work on zoonotic diseases and parasites in Alaska as a research scientist working with the Arctic Health Research Center (AHRC) of the U.S. Public Health Service. He even held a position in the University of Alaska system from 1967-1975 (Hoberg, 2014). He is

best known for his seminal series *Studies on the Helminth Fauna of Alaska* that catalogs helminthic parasites in Alaska. Although his work was mainly concerned with mammalian and avian parasites, his research touched on amphibian parasites in Alaska as well (Rausch and Williamson, 1959).

This thesis adds to the rich tradition of these pioneering scientists by examining the ecophysiology of wood frogs and their parasites in winter. I ask how the wood frog survives winter, how this affects parasites, and what this means for parasites in the Arctic as the environment warms. There are three significant contributions to physiology in this thesis. I establish new limits to freeze-tolerance in vertebrates, I describe a method for freezing wood frogs that mimics field conditions, and I examine how host overwintering physiology can be used by parasites for their winter survival.

In the Arctic, winter is marked by low resource availability and extreme freezing temperatures. Parasites in the Arctic must be able to either overwinter in their host, as a free-living larval stage, or be reintroduced every summer (Wharton, 1999). Special challenges arise for parasites when their host is an ectotherm since winter may mean surviving below freezing conditions. Ectotherms that overwinter in these conditions must either find thermal refugia that remain above freezing (such as a large body of water) or cope with a body temperature ($T_b$) below freezing. To survive $T_b < 0°C$ ectotherms can either be freeze-avoidant or freeze-tolerant. Freeze-avoidant animals cannot survive any internal ice formation. Supercooled animals overwinter in an unfrozen state at temperatures below their equilibrium freezing point, defined by the concentration of osmolytes in their body fluids. Any contact with ice nucleators such as dirt or ice particles will initiate ice formation and cause the animal to freeze and die. Freeze-tolerant animals are capable of surviving extracellular ice formation. Intracellular ice formation is lethal. These animals produce cryoprotectants to reduce damage due to the concentrations of solutes that follows from the freezing of extracellular fluids. Cryoprotectants are miscible, low molecular weight compounds such

as glucose, glycerol, and urea. Parasites in both freeze-avoidant and freeze-tolerant ectotherms must be adapted to these differences in their host's seasonal physiology, with freeze-tolerance appearing to be the more difficult environment for parasites.

To examine how host overwintering physiology influences parasite winter survival, the overwintering physiology of the host must be well understood and manipulatable in an experimental laboratory setting. The first two chapters in this thesis investigate the overwintering physiology of wood frogs in the field and develop a laboratory method for mimicking the field conditions that produce the levels of cryoprotectant production that occur in free living frogs. The third chapter then examines how *Ribeiroia ondatrae* may survive in a frozen wood frog.

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## Chapter 1 Wood frog adaptations to overwintering in Alaska: New limits to freezing tolerance.[1]


### Abstract

We investigated the ecological physiology and behavior of free-living wood frogs (*Lithobates [Rana] sylvaticus)* overwintering in Interior Alaska by tracking animals into natural hibernacula, recording microclimate, and determining frog survival in spring. We measured cryoprotectant (glucose) concentrations and identified the presence of antifreeze glycolipids in tissues from subsamples of naturally freezing frogs. We also recorded behavior of wood frogs preparing to freeze in artificial hibernacula, and tissue glucose concentrations in captive wood frogs frozen in the laboratory to -2.5°C. Wood frogs in natural hibernacula remained frozen for 193 ±11 consecutive days and experienced average (Oct-May) temperatures of -6.3°C and average minimum temperatures of-14.6±2.8°C (range -8.9 to -18.1°C) with 100% survival (n=18). Mean glucose concentrations were 13-fold higher in muscle, 10-fold higher in heart, and 3.3-fold in liver in naturally freezing compared to laboratory frozen frogs. Glycolipid antifreeze was present in extracts from muscle and internal organs, but not skin, of frozen frogs. Wood frogs in Interior Alaska survive freezing to extreme limits and durations compared to those described in animals collected in southern Canada or the U.S. Midwest. We hypothesize that this enhancement of freeze tolerance in Alaskan wood frogs is due to higher cryoprotectant levels that are produced by repeated freezing and thawing cycles experienced under natural conditions during early fall.


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[1]Don Larson, Luke Middle, Henry Vu, Wenhui Zhang, Anthony S. Serianni, John Duman, and Brian M. Barnes. 2014. Wood frog adaptations to overwintering in Alaska: New limits to freezing tolerance. *Journal of Experimental Biology* 217: 2193-2200; doi: 10.1242/jeb.101931

## Introduction

Freeze tolerant amphibians (those able to survive freezing) freeze at high, sub-zero temperatures to control the rate of extracellular ice formation and permit time to synthesize and distribute cryoprotectants that lessen cellular damage caused by desiccation (Layne et al., 1990; Storey and Storey, 1996). Cryoprotectants can also help increase survival after freezing by preventing intracellular ice formation, stabilizing membranes and macromolecules, and serving as antioxidants, metabolic substrates, and metabolic regulators (Storey and Storey, 1996). An additive, protective effect of cryoprotectants is suggested, since loading cells with glucose and urea reduces water loss, stabilizes cells, and increases survival after freezing (Costanzo and Lee, 2013).In addition to low molecular mass cryoprotectants, a high molecular mass xylomannan-based antifreeze glycolipid (AFGL) with thermal hysteresis activity is present in certain freeze tolerant organisms including insects such as the Alaskan beetle, *Upis ceramboides* (Walters et al., 2009), a plant, and a European frog, *Rana lessonae* (Walters et al., 2011). Most of the AFGL is present on the cell membranes, and therefore its function in these freeze tolerant species appears to be to prevent the lethal propagation of extracellular ice across the cell membrane into the cytoplasm. AFGL in *R. lessonae* also inhibits potentially damaging recrystallization of ice in the extracellular fluid.

Overwinter conditions of minimum temperature and the duration of subzero temperatures vary by location, but most freeze tolerant amphibians are believed to experience temperatures near 0°C, with only brief periods (< 1 week) of below freezing temperatures (Sinclair et al., 2013; Costanzo and Lee, 2013), although there are few descriptions of field hibernacula microclimate (Costanzo and Lee, 2013). The wood frog (*Lithobates [Rana] sylvaticus*) is a well-studied freeze tolerant amphibian that uses glucose and urea as cryoprotectants, with urea having an additional role in metabolic suppression (Costanzo and Lee, 2013). Most studies of this species have focused on

Midwest United States and southern Canada populations, which are near the southern limits of the wood frog range. Northward, the wood frog range extends above the Arctic Circle with limits in Alaska close to the Brooks Range and to the Arctic Ocean in western Canada (Martoff and Humphries, 1959).

Lower lethal temperatures in wood frogs have been reported as near -7°C (Layne et al., 1998), with a recent account, however, of survival of frogs from Alaska cooled to -16°C in the laboratory (Costanzo et al., 2013). In subarctic Interior Alaska, wood frogs overwinter in the subnivean space covered by duff and leaf litter (Kirton, 1974), where temperatures can remain below freezing for over 6 months with minima near –20°C (Barnes et al., 1996; Sformo et al., 2010). These extreme temperatures combined with previously reported limits to freeze tolerance would suggest that high mortality of wood frogs occurs in Interior Alaska. Our interest was in determining the conditions wood frogs naturally experience while overwintering near the northern limit of their distribution in Alaska, their duration of freezing and rates of survival over two winters, and their behavioral and physiological responses, including measuring levels of cryoprotectant accumulation in tissues and testing for the presence of antifreeze glycolipids.

## Results

### Selection of hibernacula

Free-living wood frogs (n=18; body mass 14±1.2 g) prepared for overwintering were found in early September covered with leaves in shallow depressions (forms) 4-10 cm deep within the organic soil located near the edge of spring breeding ponds (Fig. 1.1). The average distance of wood frogs from the pond edge was 710±821 cm (range 80-2250 cm). When sex was known, females averaged 124 cm and males averaged 1190 cm from the pond (not significant) (Table 1.1).

Wood frogs filmed after being placed in an outdoor soil and leaf filled enclosure at -5°Ccontinued to move until just before freezing initiated. Wood frogs burrowed under leaves and created forms by laterally rotating in the soil. If we uncovered wood frogs by removing leaves, they would relocate and create a new, covered form (see supplemental video).

**Freezing conditions and overwinter survival**

All 18 free-living wood frogs in natural hibernacula in April 2001 and 2002 survived winter. We considered that wood frogs began to freeze when soil temperatures were below -1.6°C (see below) and thawed when temperatures were above -0.16°C, based on the melting point of frogs determined by Sinclair et al., (2013). Combining data from both years, temperatures of wood frogs decreased below -1.6°C between October 10 and 25 and first warmed above -0.16°C in spring between April 19 and 9 May; thus, wood frogs were below their freezing point for, on average, 193 ±11 days (range 175-218 days). Between October and May average temperatures experienced by individual wood frogs ranged from -3.9°C to -8.4°C, with a grand mean of -6.3°C. Minimum temperatures experienced by frogs ranged from -8.9°C to -18.1°C, with an average minimum of -14.6±2.8°C. Minimum microhabitat temperatures were usually reached in December even though the lowest air temperatures occurred on January 24, 2001 and February 8, 2002 (Table 1.1 and Fig. 1.2 and 1.3).

Wood frogs freezing in outdoor enclosures in 2011 and 2012 experienced a minimum temperature of -22°C on November 22, 2011and -17.5°C on December 8, 2012. These wood frogs were frozen for 50 days before being sampled for tissue glucose concentrations.

Exotherms following nucleation of ice in wood frogs were observed in animals (all male; body mass 11.9±1.3 g) at an average temperature of -1.12±0.28°C (n=15) in 2011 and -1.14±0.34°C (n = 15) in 2012.The temperature at which exotherms occurred decreased with date with the

regression significant in 2012 ($r^2$=0.21, $F_{1,16}$ =9.428, $P$<0.05; Fig. 1.4) but not in 2011 ($P$=0.10; data not shown). Rates of cooling under natural conditions measured from 0.5°C until nucleation ranged among frogs from 0.35 to 1.60 °C per hour.

In early October of all years wood frogs experienced multiple (average of 12, range 10-17) and mostly successive cycles of freezing soil temperatures during night and thawing soil temperatures that lasted from 2-32 h during day (Fig. 1.4).

**Glucose concentrations**

Glucose concentrations in tissues of free-living wood frogs sampled while frozen in April 2001 and 2002 and in wood frogs held in outdoor enclosures and sampled frozen in December 2011 and 2012 were not significantly different (all comparisons, $P$>0.20), and therefore values for each tissue were combined over years. Naturally frozen wood frogs had glucose concentrations (mean±SEM) in liver of 788±98.8 μmol $g^{-1}$ fresh weight, in leg muscle (gracilis major) of 299±32.2 μmol $g^{-1}$ fresh weight, and in heart of 596±50.9 μmol $g^{-1}$ fresh weight. These tissue glucose concentrations were significantly higher than corresponding values in liver ($F_{2,53}$= 25.4, $P$<0.0001), heart ($F_{2,53}$= 25.4, $P$<0.0001), and leg muscle ($F_{2,53}$= 25.4, $P$<0.0001) measured in laboratory frozen wood frogs. Laboratory frozen wood frogs had glucose concentrations in liver 238±40.2 μmol $g^{-1}$ fresh weight, in muscle 23.8±5.6 μmol $g^{-1}$ fresh weight, and in heart 60.5±16.2 μmol $g^{-1}$ fresh weight. There were no significant differences between mean glucose concentrations in tissues from laboratory frozen wood frogs held at -2.5°C for durations of 24, 30, 74, and 144 hrs ($P$>0.10). Both laboratory and naturally frozen wood frogs had significantly higher ($P$<0.0001) glucose concentrations in corresponding tissues than in unfrozen, control wood frogs where average concentrations were 40.2±8.9 μmol $g^{-1}$ fresh weight in the liver, 5.4±1.5 μmol $g^{-1}$ fresh weight in the muscle, 1.9±0.6 μmol $g^{-1}$ fresh weight in the heart. (Fig. 1.5, Table 1.2).

**Antifreeze glycolipid**

R1 samples (containing solute that was in solution and/or weakly bound to the cell membranes, see Materials and Methods for details) extracted from both the skeletal muscle and organ fractions showed thermal hysteresis (TH) activity, as did the R2 (more strongly bound membrane associated) organ samples, indicating the presence of either antifreeze protein or antifreeze glycolipid (Table 1.3). In contrast, the skin had minimal TH. Most of the TH activity was extracted with the initial R1 buffer, but a lesser amount of activity was present in the organ R2 sample as well, indicating that at least some of the activity was associated with the cell membrane. Overnight treatment of the muscle R1 sample with trypsin did not reduce the level of TH (Table 1.3), suggesting that the activity was not due to an antifreeze protein, and therefore perhaps resulted from an antifreeze glycolipid. Also, elimination of TH in the organ R1 sample by xylanase treatment (Table 1.3) indicated that AFGL was likely responsible for the TH. This was confirmed by the 600-MHz $^1$H NMR spectrum of the R1 sample from frog muscle shown in Fig. 1.6 (A, full spectrum; B, expanded region containing saccharide signals). For comparison, the same expanded region of the $^1$H NMR spectrum of the AFGL isolated from the freeze tolerant beetle *Upis ceramboides* is shown in Fig. 1.6C. While the saccharide regions in Fig. 1.6B and 1.6C do not match with regard to relative signal intensities, there is good correspondence between the two spectra with regard to signal positions, as illustrated for the downfield anomeric proton signals $H1_M$ and $H1_X$, and the up field $H5b_X$ and $H2_X$ signals. These data indicate that the frog sample is chemically similar to the *U. ceramboides* AFGL, namely, both are composed of ßMannose and ß-Xylose residues in 1→4-linkage. In addition, in the wood frog sample, signals are observed near 1.5 ppm (Fig. 1.6A), indicating the presence of $CH_2$ groups and suggesting the possibility that the sample contains a lipid component as proposed for the *U. ceramboides* AFGL. NMR spectra of muscle R2 and organ R1 and R2 samples

(not shown) were similar to that of the muscle R1 sample shown in Fig. 1.6. In addition, signals consistent with the presence of protein did not appear in the NMR spectra, adding further evidence of the absence of AFP in the sample.

**Discussion**

Our study is the first to examine the ecological physiology, biochemistry, and behavior of freeze tolerant wood frogs overwintering under natural conditions. We describe movements of wood frogs preparing to overwinter, the locations and microclimates of their hibernacula, and tissue cryoprotectant concentrations in free-living wood frogs near the northern limits of their species' distribution in Interior Alaska. This study is also the first to report the presence of antifreeze glycolipid in wood frogs. We found that both freeze tolerance endurance and minimum temperatures experienced by Alaska wood frogs are more extreme than previously established. We also demonstrate wood frogs freezing under natural conditions accumulate much higher tissue concentrations of glucose compared to levels measured in captive wood frogs frozen under standard laboratory protocols.

In Interior Alaska, wood frogs overwintered on the forest floor within mixed spruce and birch woods. Wood frogs were located within soil in small forms 4-10 cm below the top of the leaf litter, covered with decaying leaves and branches. Disturbed wood frogs relocated to a new form; Kirton (1974) also observed wood frogs relocating after disturbance in early fall. Overwintering wood frogs were found close to breeding ponds (0.8-2.2 m from pond's edge) with females tending to overwinter closer to ponds than males. This finding, although not statistically significant, supports previous reports of wood frogs in which males were located in spring closer to breeding sites than were females (Regosin et al., 2003)

In artificial enclosures wood frogs moved underneath the leaf litter and pressed the soil down by rotating their body laterally to create a form within the dense, moist soil (supplemental video). In comparison, the Couch's Spadefoot Toad (*Scaphiopus couchii*) uses its clawed hind legs to burrow into sandy substrate (Mayhew, 1965). Wood frogs, without claws, may rotate instead of dig since the soil likely requires less effort for the wood frog to compact than displace. Wood frogs were active at sub-zero temperatures and capable of movement until ice nucleation was initiated. This species is characterized by tolerance to cold, showing rapid embryonic development at low temperatures (Moore, 1939).

Duration of freezing survival in wood frogs in Interior Alaska was much longer than that reported from other studies, with temperatures within wood frog hibernacula remaining below the freezing point for up to 218 days, over 7 months, with 100% survival (Table 1.1). In contrast, a laboratory study with Alaskan wood frogs placed a limit of 2 months for freezing endurance with 50% survival (Costanzo et al., 2013). To our knowledge, no other study has measured temperatures of free-living wood frogs in their hibernacula; however, in the warmer climate of Ontario, Canada, Sinclair et al., (2013) recorded winter temperatures in the subnivean space where wood frogs had been observed and concluded that conditions would result in wood frogs being frozen for up to76 consecutive hours during each freezing incident for a total of only 11-13 days frozen over the course of the winter.

Until recently, lower lethal temperatures of wood frogs were reported as approximately -7°C (Layne et al., 1998). Costanzo et al., (2013) extended this limit to -16°C for Alaskan frogs, and here we further extend this to -18.1°C, the minimum temperature experienced by wood frogs overwintering under natural conditions (all of which survived). It is likely that wood frogs can survive still lower temperatures, at least to -20°C, that regularly occur below the snow in Interior

Alaska (Sformo et al., 2010).Wood frogs selected for glucose determinations in 2011 were exposed to a minimum temperature of -22°C, and although these were not examined for survival, tissue glucose concentrations were the same as wood frogs that survived freezing, and we believe that they were alive when sampled. In2001 and 2002, animals surviving to spring under natural conditions experienced at least -8.9°C with the average minimum temperature of-14.6±2.8°C (Table 1.1). While hibernacula temperatures remained relatively stable over the winter, air temperatures fluctuated greatly and reached minima of -36.8°C in 2001 and -40.7°Cin 2002 (Fig. 1.2 and 1.3). Relative warmth and stability in hibernacula temperatures were the result of the insulation created by air trapped in overlying leaves and snow cover. In both years, snow depth increased over the winter resulting in all but two minimum temperatures occurring in December, although the lowest air temperatures occurred later. Soil temperatures (5cm below the surface; Environmental Data Center Team, Toolik Field Station, 2013) measured on the North Slope of Alaska 250 km north of the wood frog distribution limits usually are not lower than hibernaculum temperatures in Interior Alaska, suggesting that minimum temperatures in winter do not limit the northern range of wood frogs. Their northern range in Alaska may instead be limited by other abiotic conditions such as the geographical barrier of the Brooks Range or prolonged low water temperatures in breeding ponds that may prevent complete metamorphosis of tadpoles in summer (Martoff and Humphries, 1959; Herreid and Kinney, 1967).

Enhanced tolerance to freezing has been previously demonstrated in Alaskan wood frogs in a preliminary field study (Middle and Barnes, 2001) and recently in the laboratory (Costanzo et al., 2013), although results presented here extend limits in both minimum temperature and especially duration of freezing. The physiological basis of this profound cold tolerance may lie in the high levels of glucose accumulation in tissues, effects of additional cryoprotectants such as urea

(Costanzo et al., 2013), the presence of AFGL, or likely a combination of these and other factors that create protection from extracellular ice formation and accompanying desiccation.

Glucose concentrations in liver, heart, and leg muscle from naturally freezing wood frogs were much higher than levels measured in corresponding tissues from wood frogs frozen in the laboratory in this and in other studies (Fig. 1.5 and Table 1.2). Despite being cooled at rates of 0.05 and 0.5°C/h, slower than the rates observed under natural conditions (as high as 1.6 °C/h), Alaskan wood frogs frozen in the laboratory accumulated glucose to levels that were only 22-40% in liver, 6-31% in heart, and 7-20% in thigh muscle as compared to those in corresponding tissues in wood frogs that froze outdoors (Table 1.2). Wood frogs from Ontario, Canada and the U.S. Midwest frozen in the laboratory had glucose concentrations that were 8-62% in liver and 3-10% in heart of those measured in naturally freezing Alaskan frogs (Table 1.2). Alaskan wood frogs may accumulate these high levels of glucose in their tissues by initially storing more glycogen as a source, releasing more glucose when freezing, or through repeated episodes of freezing-stimulated release of glucose, coupled with decreased rates of glucose uptake or loss during thaw.

Wood frogs collected in Interior Alaska indeed accumulate very high levels of glycogen in fall, approximately 3.5-fold the concentrations in liver and muscle measured per gram of frog compared to wood frogs collected in Ohio (Costanzo et al., 2013). Despite these large differences in the relative amount of glycogen stored, Alaskan and Ohio wood frogs were similar, however, in how much glucose they mobilized into liver, heart, and muscle 48 h after freezing is initiated, when freezing occurs via a linear decrease in temperature (Costanzo et al., 2013). This result suggests that it is not just the large stores of glycogen that account for the high levels of mobilized glucose in naturally frozen Alaskan frogs, but also the pattern of freezing that includes multiple freeze thaw cycles.

We hypothesize that it is the pattern of freezing under natural conditions that includes multiple freezing and thawing cycles that causes the high concentrations of glucose that accumulate in tissues of Alaskan wood frogs, and that these high glucose concentrations contribute to the enhanced tolerance to cold that we have demonstrated. Beginning in early October of each year, soil temperatures in wood frog hibernacula decreased below -0.5 °C most nights, and exotherms, indicative of the initiation of freezing, occurred shortly followed by thawing conditions during most days that lasted for 12.2 h, on average. Wood frogs overwintering under natural conditions experienced as many as 17 mostly successive freezing and thawing episodes before temperatures decreased and remained below freezing until spring.

The decrease in the temperature at which successive exotherms occurred (Fig. 1.4) could be explained by the increase in overall solute concentration that occurs in frogs as they accumulated glucose, since increasing osmolarity decreases the supercooling point in fluids (Zachariassen, 1985). The decrease in exotherm temperatures of frogs over time was statistically significant in only one of the two years we measured, however.

If each exotherm results in a stimulus for conversion of stored glycogen to glucose and if glucose accumulates in tissues due to low rates of loss or re-synthesis into glycogen during thaw at low temperatures, then successive freeze thaw cycles in wood frogs should result in higher and higher tissue concentrations of glucose. Inoculative nucleation of freezing detected in skin of wood frogs is a required stimulus for the breakdown of liver glycogen stored into glucose, which is then distributed throughout the body (Storey and Storey, 1986), and consecutive 2 day cycles of freezing and thawing resulted in higher glucose levels than in controls (Storey and Storey, 1988) although not to the levels shown in naturally freezing frogs in this study. Also, glucose is indeed retained in tissues at higher levels after thaw in Alaskan relative to southern populations of wood frogs. Successive

freeze thaw cycles lead to accumulation of tissue glucose concentrations because glucose synthesis following freezing is faster than reconversion of glucose to glycogen following thaw (Storey and Storey, 1986). Glucose levels remained at 20-50% (average 30%) of maximal values reached over 48 h of freezing 5 days after thaw in plasma, brain, liver, heart and muscle of Alaskan frogs compared to values of 2-30% (average 9%) in Ohio frogs (Costanzo et al., 2013). Levels of distributed glucose may change little during the daily intervals of slightly above freezing temperatures experienced by free-living frogs in Alaska that lasted only about 12 h before another stimulus for glucose release occurred at night. Whether wood frogs from southern populations experiencing successive freezing and thawing stimulus can accumulate as high a level of glucose in tissues as Alaskan frogs do and whether this would enhance their tolerance to freezing is not known.

Freezing tolerance in wood frogs may also be enhanced due to the presence of antifreeze glycolipids in their membranes and tissues. Ice purified extracts derived from homogenized samples of skeletal muscle and internal organs of naturally overwintering wood frogs demonstrated a level of TH activity that is usually associated with antifreeze proteins. However, trypsin treatment did not affect the TH activity (Table 1.3), suggesting that the TH is not dependent on a protein. In contrast, treatment of the R1 organ sample with endo ß-(1→4) xylanase eliminated the TH, as was the case with the antifreeze glycolipid from the freeze tolerant Alaskan beetle, *U. ceramboides* (Walters et al., 2009). Also, NMR spectra of wood frog antifreeze showed signals with similar positions to saccharides of AFGL from *R. lessonae*, as well as from various insects and a plant, indicating a backbone consisting of ßMannose and ß-Xylose residues in 1→4-linkage (Walters et al., 2009, 2011). While lipid signals were also present in the NMR spectra, perhaps indicating the presence of fatty acids that anchor the AFGL in membranes, the NMR spectra did not exhibit amino acid signals consistent with protein. Consequently, wood frogs appear to have an AFGL similar to those

described in other species. While the function(s) of the AFGLs are not known, they may inhibit damaging recrystallization of ice in the extracellular fluid where ice is present and prevent propagation of extracellular ice across the cell membrane and into the cytoplasm that is lethal in most cells of freeze tolerant animals.

Limitations to duration of freeze tolerance and minimum freezing temperature include extracellular recrystallization, metabolic demand, waste accumulation, intracellular ice formation and desiccation (Knight and Duman, 1986; Storey and Storey, 1988; Layne et al., 1998). Wood frogs overwintering in Interior Alaska must prevent intracellular ice formation and limit extracellular recrystallization for over 6 months; they may accomplish this despite very low temperatures by accumulating high levels of intracellular cryoprotectants and production of antifreeze glycolipid. Further, the low temperatures wood frogs experience should minimize rates of metabolism so that waste accumulation and hypoxia do not constrain freeze tolerance. Kirton (1974) observed that juvenile wood frogs that did not survive overwintering were desiccated at the beginning of spring. We observed similar desiccation due to sublimation when holding frozen wood frogs in a laboratory setting (Larson and Barnes, unpublished). Wood frogs frozen in moist environments, such as wet moss, are able to maintain a greater volume of body water than wood frogs frozen in dry environments (Churchill and Storey, 1993). Forms created under leaves should create a moist environment for overwintering, and therefore wood frogs may hibernate underneath leaf litter to minimize rates of water loss, as well as to buffer the extremes and variability of air temperatures.

Our results demonstrate that Alaskan wood frogs can survive being frozen for up to 7 months with minimum temperatures below -18°C. Only the Siberian salamanders *Salamandrella schrenckii* and *S. keyserlingii*, which endure 4-5 months frozen with survival of individuals to -35°C (Berman et al., 1984; Berman et al., 2010), are comparable to capabilities of North American wood

frogs. Whether the extremes in freezing tolerance demonstrated here in northern compared to more southern populations of wood frogs are due to differences in glycogen concentrations and acclimatization and patterns of temperature change during freezing or due to differences in their genetics, and thereby represent evolutionary change, awaits further study.

## Materials and Methods

### Field and laboratory studies

We studied wood frogs over the course of four winters. Initially we collected adult wood frogs by hand in September 2000 and 2001 by searching open fields near known breeding ponds in birch and spruce boreal forest around the Fairbanks North Star Borough (64.8 ° N, 147.8 ° W), (n=8, 10, respectively). We attached radio transmitters (model V1G102A with 10 cm whip antenna, Sirtrack, Havelock North, New Zealand; weight 0.95 g) with cyanoacrylate glue to the back of individual wood frogs that weighed at least 12 g. Tagged wood frogs were held overnight and released the following day at their collection sites. Using radio receivers and Yagi antennas (Telonics, Inc, Mesa, AZ, USA), we re-located wood frogs daily until they stopped moving. In late September 2000 and 2001, we located 4 additional wood frogs (2 each year) within their hibernacula by raking the leaf litter near the edge of breeding ponds. A temperature logger probe (Hobo Pro, Onset Corp., Bourne, MA,USA) was positioned between each wood frog's ventrum and the surrounding soil, and a wire-mesh cage (1 cm squares, 20x20x20cm) was placed over each wood frog to prevent disturbed wood frogs from relocating. Air temperature was recorded with a temperature logger probe placed 2 m above the duff layer located near overwintering frogs. Temperatures were recorded every 5 minutes until wood frogs emerged from hibernation the next spring. Snow depth was taken from daily recordings for the nearby Fairbanks International Airport (5.5 km from the study site). Beginning in early April, we assessed wood frogs for movement each day. Wood frogs were

considered thawed and alive when they moved from the small depressions in the duff within which they overwintered. Four wood frogs were collected in early April (2001) and double pithed before thawing; tissues from these wood frogs were collected for glucose determinations.

In 2011 and 2012, we also collected 15 male wood frogs each year from July to August in the Fairbanks North Star Borough. Each wood frog was swabbed and determined to be negative for chytrid fungus with qPCR (Pisces Molecular, LLC, Boulder, CO, USA) and transferred to 1x2.4x2.4m outdoor enclosures in the Biological Reserve (64.8°N, 147.8°W) at the University of Alaska Fairbanks. The enclosures were located in a birch and spruce forest with conditions similar to the natural habitat of wood frog overwintering locations. Pools of water were present in the enclosures, and wood frogs were fed crickets and wingless fruit flies daily until temperatures decreased below freezing in mid-September. We surrounded each dormant wood frog with a 20x20x20cm wired cage and placed a temperature logger (Tidbit, Onset Computer, Bourne, MA, USA) in contact with each frog. Temperatures were recorded every 30 seconds. Frozen wood frogs were collected on December 12$^{th}$, double pithed, and their tissues collected for glucose determinations.

We filled a plastic pool-container (121 cm diameter) with 10 cm soil and 5-10 cm of leaf litter and placed the pool in an environmental chamber held at -5°C. In early September, 2012, two naturally acclimated wood frogs from our outdoor enclosure were released into the leaf litter and filmed with 2 cameras for 18 hours as they became frozen.

We collected 22 adult male wood frogs in August 2001 and we acclimated them for 1 week in a refrigerator set at 5°C. Wood frogs to be frozen were placed in 50 ml plastic containers with a type T thermocouple placed against their ventrum. A thermocouple thermometer (Iso-Thermex, Columbus Instruments, Columbus, OH, USA) recorded temperatures. We cooled wood frogs in

their containers in an alcohol-water bath (Neslab ULT-80, Waltham, MA, USA) at a constant rate of 0.5°C per hour from 1°C to -2.5°C. Wood frogs were nucleated with ice at -1°C and an exotherm indicating freezing was observed. Wood frogs were held at -2.5°C for 24, 30, 74, and 144 hours (n = 3, 3, 6, 3). Frozen and unfrozen, control (n = 7) wood frogs were pithed and tissues were collected for glucose determinations.

Liver, leg, and heart tissue were dissected from each wood frog. Tissue samples (50 mg) were homogenized with 0.6 N ice-cold perchloric acid and centrifuged. Extracts were neutralized and assayed in triplicate for glucose concentrations with a YSI-2000 analyzer, comparing with a standard solution (YSI, Inc, Yellow Springs, OH, USA)

**Screening and isolation for antifreeze glycolipids and NMR spectroscopy**

Tissues from 11 naturally frozen frogs were collected in spring 2012and shipped frozen on dry ice to the University of Notre Dame where they were held at -80°C until processed. The frogs were thawed and dissected with the tissues and organs separated and pooled into three groups: skeletal muscle (1.8 grams), skin (4.8 grams), and internal organs (remaining tissues and heart, liver, lungs, etc., but not bone; 18.0 grams). These were cut into small pieces with scissors and homogenized in 50 mMTris-HCl buffer (pH7.4) at an 8:1 ratio of volume of buffer to wet mass of tissue. The homogenized tissues were sonicated (Heat Systems-Ultrasonics, Inc. Farmingdale, NY, USA, W-385 sonicator) using the sonicator horn and three 30-second intervals (power level 3). The samples were centrifuged (10,000 g for 20 minutes at 4°C) and the supernatant (identified as the "R1" sample) and pellet separated. The pellet was extracted with the urea-based buffer from the Bio-Rad Ready Prep sequential extraction kit to solubilize lipophilic membrane bound molecules. This sample was centrifuged at 10,000g for 20 minutes at 4°C, and the supernatant dialyzed (3,500 MW cut-off, Spectrapor) for 24 hr at 4°C. This is identified as the "R2" sample. The osmolality of both

samples was adjusted to 200 mOsm with glycerol and subjected to multiple rounds of ice-affinity purification (Walters et al., 2009), a technique that utilizes the unique ability of thermal hysteresis producing antifreeze proteins and glycolipids to bind to ice rather than to be excluded from the ice crystal lattice as ice formation proceeds. Following this the samples were dialyzed against Mill-Q water for 48 hr to remove the glycerol, freeze dried, and re-dissolved in a small volume of Mill-Q water.

Freezing and melting points of the resulting samples were measured using a nanoliter osmometer (Nickell et al., 2013) to determine whether the samples displayed thermal hysteresis (TH, melting point and freezing point differ) indicative of the presence of antifreeze proteins and/or glycolipid. Subsamples that exhibited TH were treated with proteomics grade trypsin (Sigma, porcine pancreas) according to the manufacturer's directions. Loss of TH after trypsin treatment would indicate that TH resulted from antifreeze proteins. Because previously investigated antifreeze glycolipids contained xylose and were inactivated by xylanase (Walters et al., 2009; 2011), subsamples with TH were also treated with endo ß-(1→4) xylanase (Sigma, St. Louis, MO, USA from *Thermomyces languginosus*) in 50mM sodium citrate buffer, pH=5.0.

Samples with TH suspected of containing AFGLs were lyophilized then dissolved in 200 mL of 20 mM aqueous ($^2H_2O$) sodium phosphate buffer at pH 7.5 (meter reading), and the resulting solution was transferred to a 5-mm symmetrical Shigemi NMR microtube with susceptibility matched to $^2H_2O$. High-resolution 1D $^1H$ NMR spectra were obtained at 40°C on a Varian UNITYPlus 600-MHz FT-NMR spectrometer equipped with a 5-mm $^1H$-$^{19}F/^{15}N$-$^{31}P$ AutoX dual broadband probe. $^1H$ NMR spectra were collected with 1500 transients, 7670 Hz spectral windows, and ~3.0 s recycle times. Exponential line-broadening of 0.5 Hz was applied to free

induction decays prior to Fourier transformation. The final digital resolution of transformed spectra was 0.03 Hz/point. Spectra were referenced internally to the residual HOD signal at 4.800 ppm.

**Statistical inferences**

Sample means were compared using Student's t-test and analysis of variance (ANOVA) and analysis of co-variance (ANCOVA) followed by Tukey multiple comparisons tests. Linear regressions were calculated for exotherms. Mean values are reported as ±S.E.M. Significance of statistical analyses was accepted at $P<0.05$.

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Fig. 1.1. Wood frog in a naturally made overwintering form; covering leaves removed for photo.

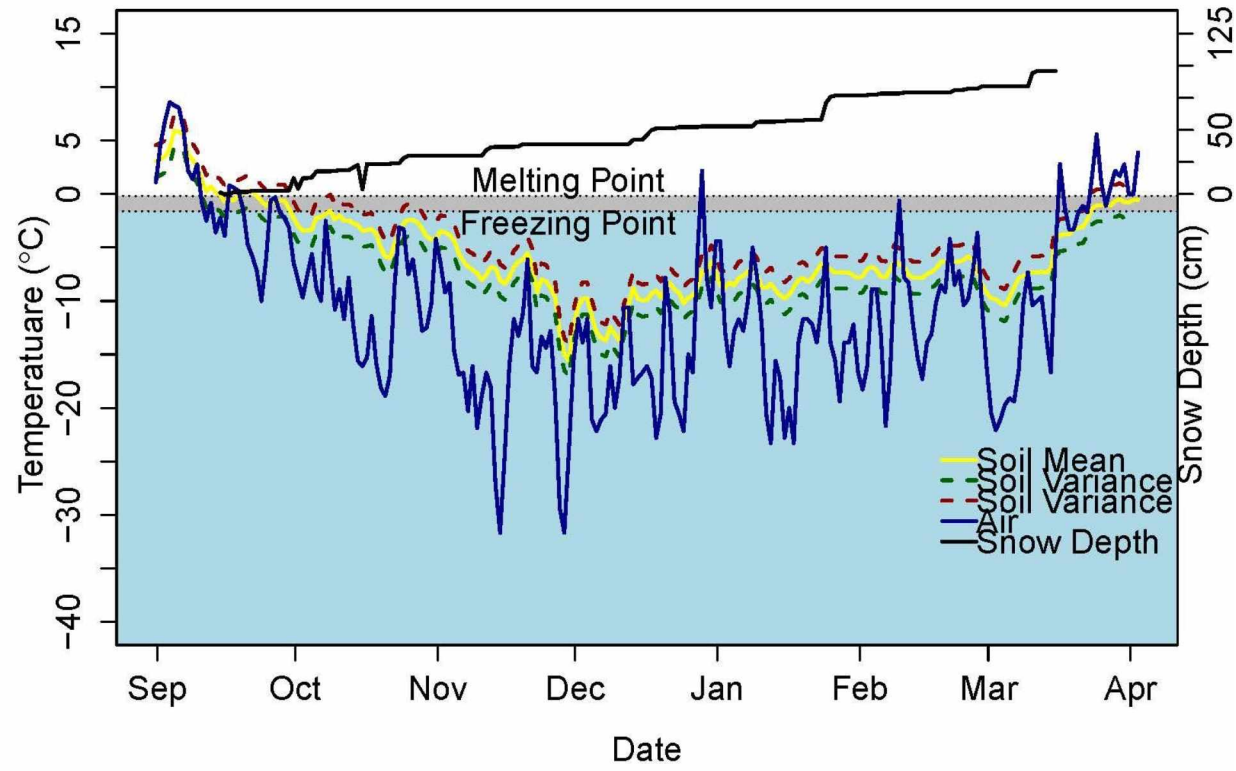


Fig. 1.2. Average daily air temperature, average daily soil temperature, daily variance (±SEM), and daily snow depth at frog hibernacula (n=8) from September 2000 to April 2001

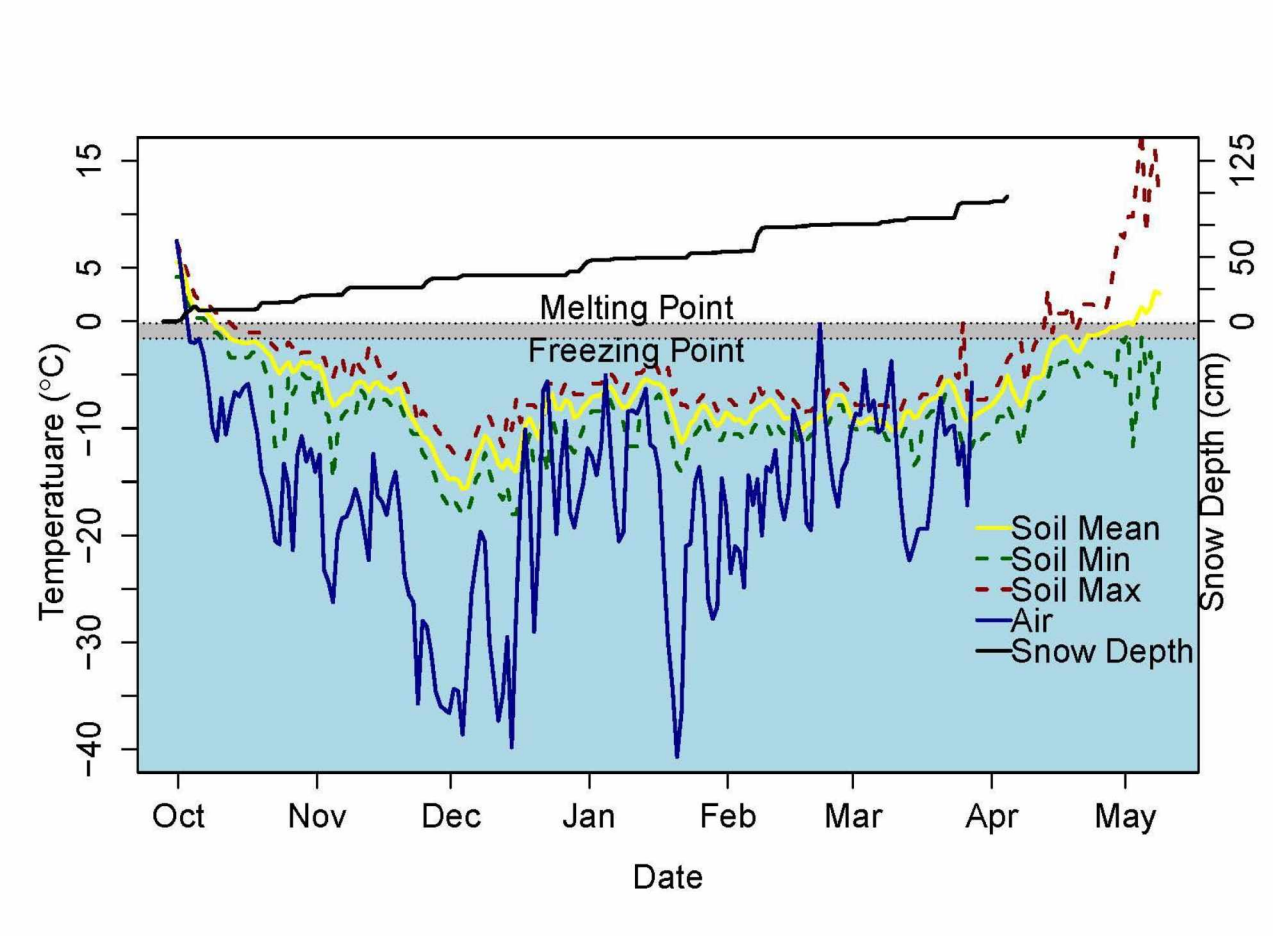


Fig. 1.3. Daily snow depth, average daily air temperature, average daily soil temperature, minimum and maximum daily temperature among frog hibernacula (n=10) from October 2001 to May 2002

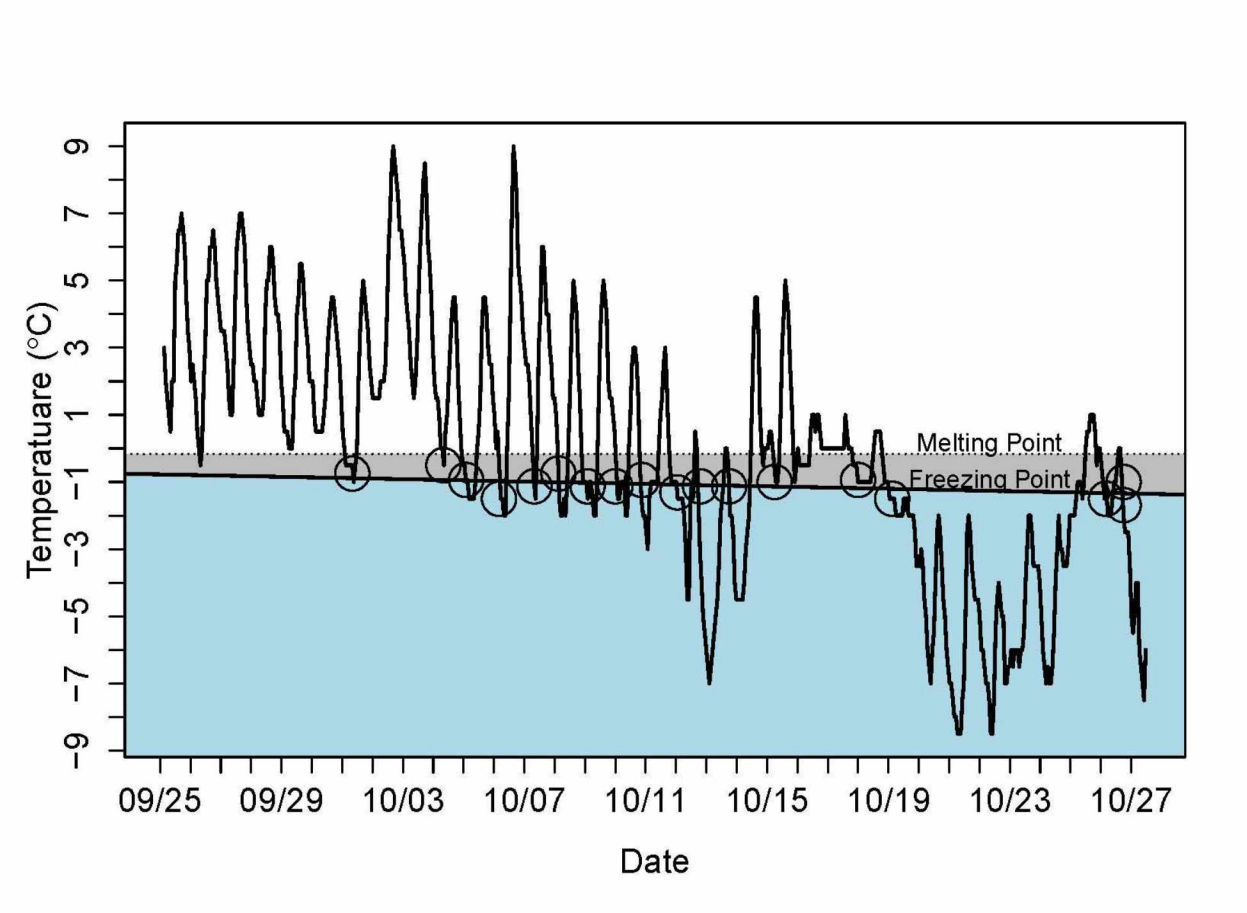


Fig. 1.4. Wood frog temperatures in a frog form recorded every 30 seconds from September 24 to October 27, 2012. Melting point is -0.16 (dashed line) (Sinclair et al., 2013) and freezing point (solid line) regression line $P<0.05$, $r^2=0.21$, $F_{1,16}=9.428$. Circles indicate observed exotherms

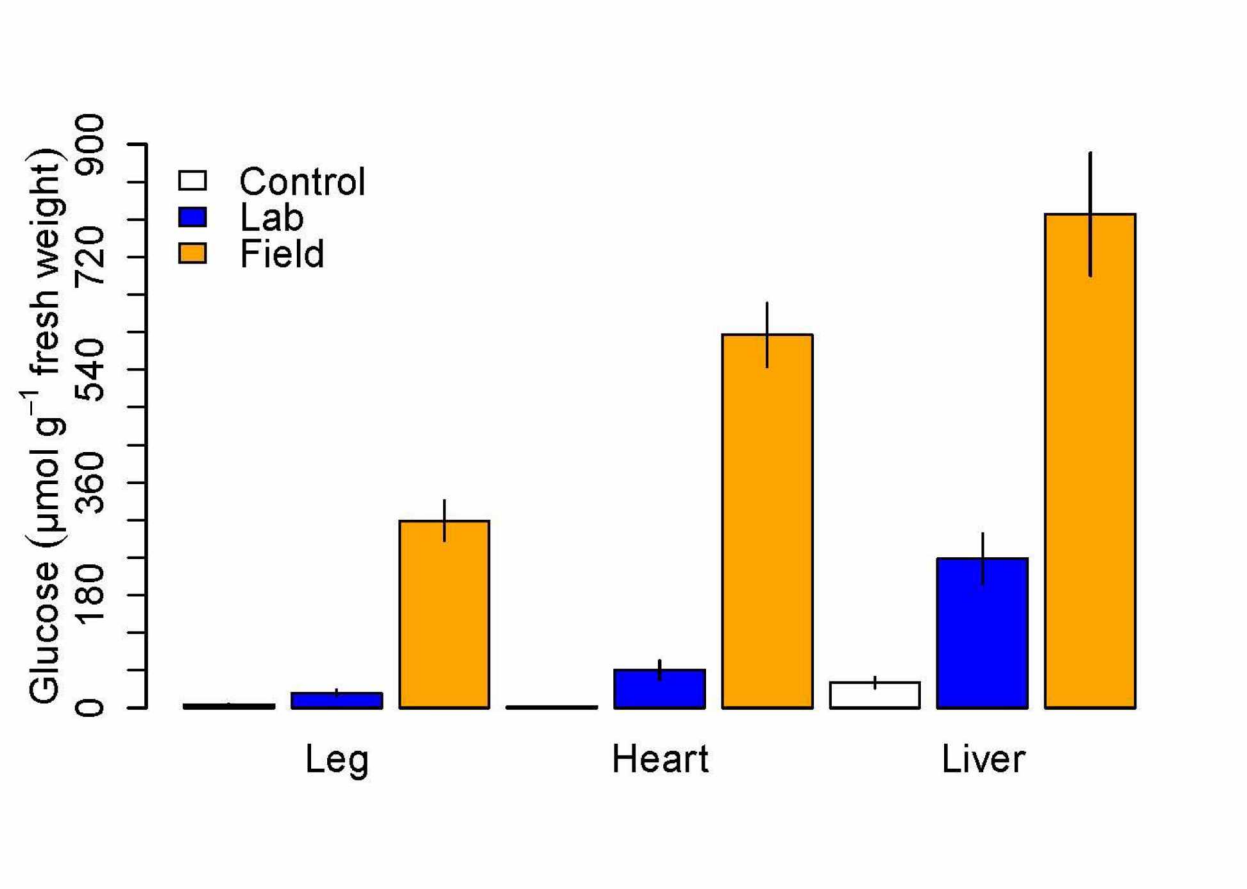


Fig. 1.5. Leg muscle, heart, and liver tissue glucose concentrations in unfrozen control, laboratory frozen, and naturally frozen wood frogs, n=7,15, 34, respectively. Concentration is represented as means and error bars are ±SEM. Body mass did not affect glucose concentrations (ANCOVA $p>0.65$) and concentrations are expressed per gram wet weight. All values vary significantly from each other [liver ($F_{2,53}= 25.4$ $P<0.0001$), heart ($F_{2,53}= 36.4$ $P<0.0001$), and leg muscle ($F_{2,53}= 15.4$ $P<0.0001$)]

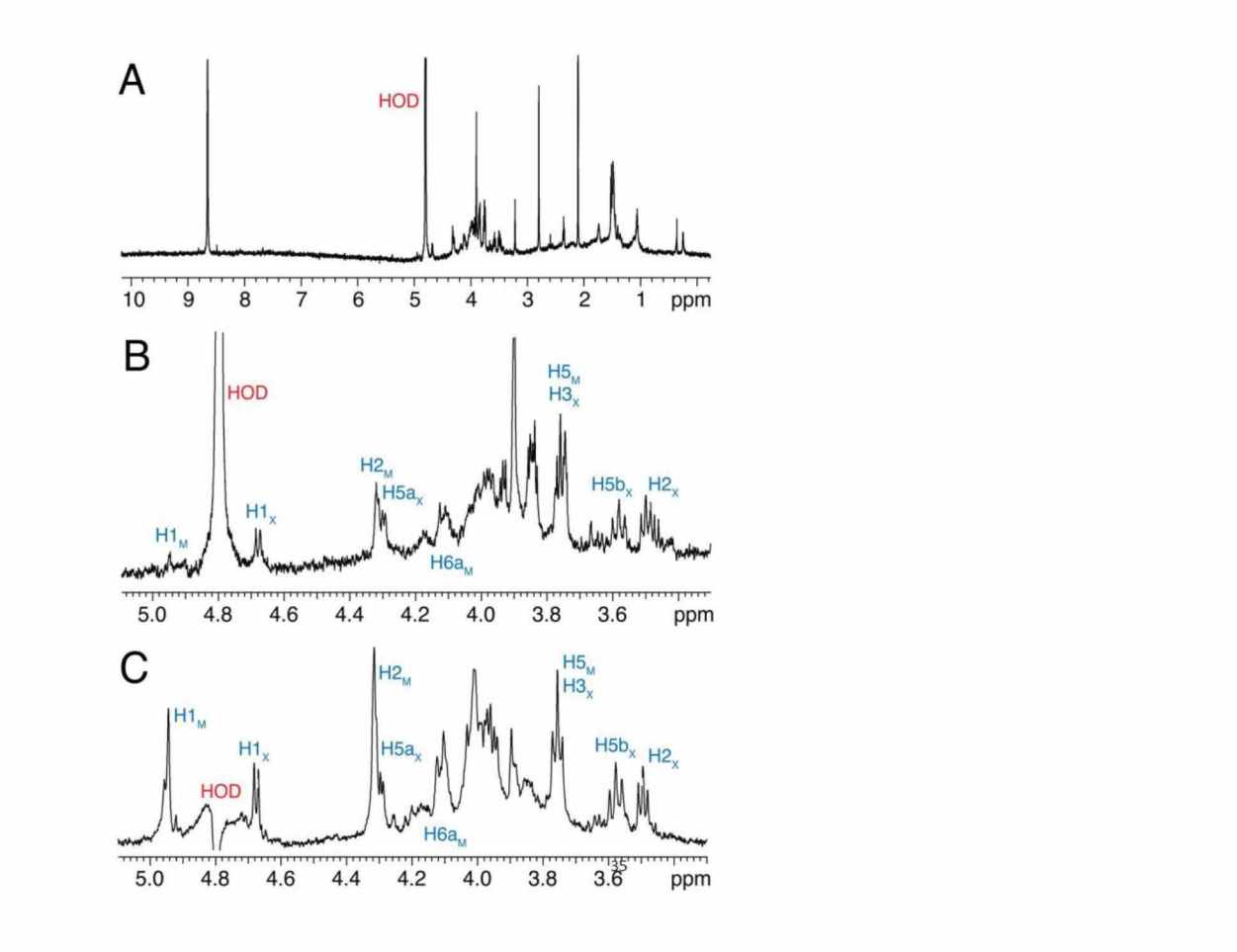


Fig. 1.6. High-resolution $^{1}$H NMR spectra of AFGL isolated from wood frog skeletal muscle (A and B) and from *Upis ceramboides* (C; data taken from Walters et al., 2009). The full spectrum of the frog AFGL is shown in (A), and an expanded region of (A) containing the saccharide signals is shown in (B). Signal assignments were made according to Walters et al., 2009. Subscripts X and M refer to the hydrogen atoms found in the ßMannose and ß-Xylose rings, respectively.

Table 11. Characteristics of natural wood frog hibernacula over 2 winters.
nr indicates not recorded.

| Year | Date of Freezing | Date of Emergence | Total consecutive days below -1.6(°C) | Mean form temperature Mean±SEM (°C) | Minimum form temperature (°C) | Date of minimum temperature | Distance to pond in spring (cm) | Sex |
|---|---|---|---|---|---|---|---|---|
| 2000 | 19 Oct | 29 April | 191 | -6.0±0.02 | -16.0 | 16 Dec | nr | nr |
| 2000 | 18 Oct | 25 April | 188 | -7.6±0.01 | -12.3 | 17 Dec | 175 | nr |
| 2000 | 20 Oct | 21 April | 182 | -4.9±0.02 | -14.1 | 16 Dec | 195 | nr |
| 2000 | 17 Oct | 21 April | 185 | -4.5±0.02 | -9.5 | 16 Dec | 160 | nr |
| 2000 | 18 Oct | 25 April | 188 | -3.9±0.01 | -8.9 | 17 Dec | 1150 | nr |
| 2000 | 20 Oct | 25 April | 186 | -4.5±0.02 | -10.0 | 3 Feb | 1540 | nr |
| 2000 | 22 Oct | 21 April | 180 | -5.1±0.02 | -17.4 | 4 Feb | 2060 | nr |
| 2000 | 20 Oct | 25 April | 186 | -7.6±0.01 | -16.7 | 16 Dec | 2250 | nr |
| 2001 | 12 Oct | 8 May | 207 | -8.4±0.02 | -18.1 | 8 Dec | 180 | F |
| 2001 | 11 Oct | 8 May | 218 | -7.2±0.02 | -16.0 | 20 Dec | 240 | nr |
| 2001 | 20 Oct | 3 May | 194 | -6.2±0.01 | -13.5 | 8 Dec | 140 | M |
| 2001 | 15 Oct | 6 May | 202 | -7.6±0.02 | -16.7 | 8 Dec | 90 | F |
| 2001 | 17 Oct | 3 May | 197 | -5.8±0.02 | -14.7 | 8 Dec | 120 | F |
| 2001 | 18 Oct | 9 May | 202 | -5.9±0.02 | -16.0 | 8 Dec | 80 | F |
| 2001 | 20 Oct | 2 May | 193 | -6.6±0.01 | -13.5 | 20 Dec | 120 | F |
| 2001 | 19 Oct | 29 April | 191 | -6.8±0.01 | -16.0 | 8 Dec | 150 | F |
| 2001 | 10 Oct | 9 May | 211 | -7.1±0.02 | -17.4 | 8 Dec | 2060 | M |
| 2001 | 25 Oct | 19 April | 175 | -7.6±0.02 | -16.0 | 8 Dec | 1370 | M |
| mean | | | 193 | -6.2 | -14.6 | | 710 | |

Table 1.2. Glucose concentrations (μmol $g^{-1}$ fresh weight) in liver, heart, and thigh muscle in unfrozen (control), linearly laboratory frozen, and naturally frozen wood frogs from Alaska, USA, Ohio, USA, and Ontario, Canada

| Treatment | Number Collected | Collected from | Liver μmol $g^{-1}$ fresh weight | Heart μmol $g^{-1}$ fresh weight | Thigh Muscle μmol $g^{-1}$ fresh weight |
|---|---|---|---|---|---|
| Control * | 7 | AK, USA | 40.2± 8.9 | 1.9±0.6 | 5.4±1.5 |
| Linearly * | 15 | AK, USA | 238±40.2 | 60.5±16.2 | 23.8±5.6 |
| Naturally Frozen* | 19 | AK, USA | 788±98.8 | 596±50.9 | 299±32.2 |
| Linearly Frozen+ | 6 | ON, CAN | 387.8±44.8 | 198.3±27.3 | 26.5±2.7 |
| Linearly Frozen^ | 3 | OH, USA | 63.7±14.1 | - | 9.7± 2.3 |
| Linearly Frozen# | 4 | OH, USA | 261.2±55 | 174.4±26.6 | 37.6±3.5 |
| Linearly Frozen# | 8 | AK, USA | 194.3±16 | 163±7.6 | 62.0±2.8 |

* This study +Storey and Storey, 1984 ^Irwin et al., 2002 #Costanzo et al., 2013

Table 1.3. Thermal hysteresis (TH, freezing point minus melting point) of various wood frog muscle, organ and skin samples. R1 sample contains soluble and/or weakly membrane bound TH factors. R2 contains more strongly membrane bound TH factors.

| **Sample** | **Thermal Hysteresis (°C)** |
|---|---|
| | |
| A. Muscle R1 | 0.61 |
| B. A (above) + trypsin | 0.62 |
| C. Muscle R2 | 0.02 |
| | |
| D. Organs R1 | 1.29 |
| E. D (above) diluted 1/1 with citrate buffer | 0.88 |
| F. D (above) diluted 1/1 with citrate buffer + xylanase | 0.08 |
| G. Organs R2 | 0.31 |
| | |
| H. Skin R1 | 0.10 |
| I. Skin R2 | 0.04 |

## Chapter 2 Cryoprotectant production in freeze-tolerant wood frogs is augmented by multiple freeze-thaw cycles[1]


### Abstract

Ice nucleation across the skin of wood frogs (*Lithobates sylvaticus*) rapidly induces endogenous production of glucose, a cryoprotectant necessary for freeze tolerance. In laboratory studies of freeze tolerance, wood frogs are cooled slowly, often at -0.05°C $h^{-1}$, to facilitate high cryoprotectant production and survival. Under natural conditions in Alaska, however, wood frogs accumulate maximal tissue glucose concentrations while cooling at much faster rates, -0.35 to -1.6°C $h^{-1}$, and, in addition, undergo multiple, successive freeze-thaw cycles before remaining frozen for the winter. We examined whether simulating these ecologically relevant cooling rates and repeated freeze-thaw events in captive wood frogs results in the high glucose concentrations found in naturally frozen wood frogs. We found that over successive freezing and thawing events glucose concentrations increased stepwise in all measured tissues. Short thawing periods did not result in a statistically significant decline of glucose concentrations. Wood frogs that experienced three freeze-thaw events had fresh weight glucose concentrations that approached values found in tissues of wood frogs frozen in natural conditions. Laboratory wood frogs survive frozen for 2 months, while wood frogs frozen under natural conditions survive frozen for up to 7 months at temperatures below -18°C. We hypothesize that repeated freeze-thaw cycles with rapid cooling and warming rates allow for greater survival in Alaskan wood frogs through enhanced cryoprotectant production.



[1] Don Larson and Brian Barnes. 2016. Cryoprotectant production in freeze-tolerant wood frogs is augmented by multiple freeze-thaw cycles *Physiological and Biochemical Zoology*, *89*(4), 340-346 doi.org/10.1086/687305

## Introduction

Wood frogs (*Lithobates sylvaticus* (LeConte, 1825)) are a freeze-tolerant amphibian capable of surviving up to 7 months frozen with hibernacula temperatures reaching below -18°C (Schmid, 1982; Larson et al., 2014). The wood frog's range, the most northern of all North American amphibians, extends north of the Arctic Circle in Alaska and to the Beaufort Sea in Canada (Martoff and Humphries, 1959). Near the northern limit of their range in Alaska, adult wood frogs hibernate in shallow depressions under leaf litter close to breeding ponds (Larson et al., 2014). To survive freezing, wood frogs use cryoprotectants to control internal ice formation, prevent intracellular freezing, and mitigate the effects of cellular desiccation caused by hyper-osmotic extracellular conditions (Storey and Storey, 1996; Costanzo and Lee, 2013).Wood frogs produce at least two cryoprotectants, urea and glucose (Costanzo and Lee, 2005), and a xylomannan antifreeze glycolipid found in cell membranes that may also contribute to freeze-tolerance (Walters et al., 2009; Larson et al., 2014). In preparation for winter, wood frogs increase urea concentrations in tissues (Costanzo and Lee, 2005), and by late summer urea plasma concentrations from wood frogs in central Alaska increase by 10-fold compared to early summer (Costanzo et al., 2014). Glucose, in contrast, is only synthesized in copious amounts in wood frogs as a response to ice formation across their skin (Storey and Storey, 1986). Glycogen stored in the liver is rapidly converted into glucose and distributed into cells as tissues amass extracellular ice. To achieve high survival and facilitate cryoprotective responses, wood frogs frozen in many laboratory studies are cooled at a rate of -0.05°C $h^{-1}$, since a slow cooling rate results in higher glucose synthesis and concentrations in tissues and, therefore, higher survival than faster rates of cooling (Costanzo et al., 1992; Costanzo et al., 2013). Glucose concentrations in unfrozen frogs in μmol $g^{-1}$ fresh weight are 40.2± 8.9 6, 1.9±0.6, and 5.4±1.5 in liver, heart, and gracilis major tissue, respectively (Larson et al., 2014). Freezing

Alaskan wood frogs at -0.05°C $h^{-1}$ to -2.5°C results in a marked increase in glucose concentrations in μmol $g^{-1}$ fresh weight across liver, heart, and leg tissues of 261.2±55, 174.4±26.6, and 62.0±2.8, respectively (Costanzo et al., 2013; Larson et al., 2014). In comparison, wood frogs frozen under laboratory conditions to -16°C or exposed to freeze-thaw cycles at cooling rates of -0.05°C $h^{-1}$ resulted in mean glucose concentrations in μmol $g^{-1}$ fresh weight of 743±229.0 and 437.84±105.4 in liver and heart tissues, respectively (Costanzo et al., 2015). Wood frogs collected in the field from natural hibernacula in Alaska had even higher glucose concentrations in μmol $g^{-1}$ fresh weight of 788±98.8, 596±50.9, and 299±32.2 in liver, heart, and gracilis major tissue, respectively (Larson et al., 2014).

Wood frogs in natural hibernacula in interior Alaska do not cool at slow rates; instead, cooling rates can be as fast as -1.6°C $h^{-1}$, a rate that is lethal for conspecifics from Ohio frozen in the laboratory (Costanzo et al., 1992; Larson et al., 2014). In field hibernacula during October, soil temperatures oscillate daily above and below the freezing point. In our previous study of wood frogs tracked to natural hibernacula, wood frogs averaged 12 nightly freezing events of 2-32 h followed by daily thaws before they remained frozen for up to 7 months (Larson et al., 2014). We hypothesize that these daily freeze-thaw events allow for a stepwise increase of glucose concentrations within tissues after each ice nucleation. For this hypothesis to be supported, glucose loss in tissues either through excretion, catabolism, or glyconeogenesis should occur at a slower rate during thawing periods than the rate of glycogenolysis during freezing. We believe these natural patterns of freezing may be important to the enhancement of freezing tolerance demonstrated in these Alaskan populations of wood frogs.

**Methods**

We collected 35 adult male wood frogs by hand along the Tanana River in mid to late August 2012 in the Fairbanks North Star Borough (64.8° N, 147.8° W). Wood frogs weighed 8-15 g. Only male wood frogs were used in compliance with our Alaska Department of Fish and Game collection permit, and experiments were conducted with the approval of the University of Alaska Fairbanks Institutional Animal Care and Use Committee (Protocol #259022). Wood frogs were held at 5°C under a 12 h photoperiod and provided crickets and wingless fruit flies and access to water. The experimental sampling scheme used for testing how freezing patterns altered tissue glucose concentrations is shown in Fig. 2.1. At each sampling point, frogs were euthanized with an immersion overdose of Finquel MS-222 (Argent Laboratories, Redmond, WA USA) at a concentration of 2 g $l^{-1}$ buffered with $NaHCO_3$ to a pH of 7.8 and temperature of 0°C and doubled pithed before removal of tissues. Liver, heart, and leg (gracilis major) tissues were stored frozen at -80°C and subsequently measured for percent water and glucose concentrations (details below). Wood frogs were divided into seven groups for sampling. Group 1 wood frogs (n=5) were euthanized as unfrozen controls. The remaining wood frogs were exposed in six groups (n=5 animals per group) to either a single or successive freezing and thawing cycle(s) and sampled either frozen or thawed. For freezing, frogs were placed individually in 200 ml plastic containers on top of moist moss. To record temperature a 30-gauge type T thermocouple attached to a recorder (Iso-Thermex, Columbus Instruments, Columbus, OH, USA) was placed against each wood frog's dorsum. Containers with frogs were placed in an ethanol-water bath (Neslab ULT-80, Waltham, MA, USA) with an initial temperature of 2°C; we nucleated ice formation in each frog during cooling when they reached -1.5°C by adding finely crushed ice on to their backs. All frogs were cooled and warmed at a rate of -0.58°C $h^{-1}$ and +0.58°C $h^{-1}$, respectively. We cooled wood frogs to a minimum

temperature of -5ºC and warmed them to a maximum temperature of 2ºC. Each cooling period and warming period lasted 12 h. Groups 2, 4, 6, and 7 wood frogs were sampled at -5ºC. Groups 3 and 5 were sampled at 2°C. Group 2 frogs were cooled from 2 to -5ºC over 12 h and sampled frozen. Group 3 animals were cooled from 2 to -5ºC over 12 then rewarmed to 2ºC over 12 h and sampled thawed at 24 h. Group 4 frogs experienced two freezing cycles and were sampled frozen at 36 h. Group 5 frogs were frozen and thawed twice and sampled thawed at 48 h. Group 6 frogs were sampled frozen after three freezing events over the course of 60 h. Group 7 wood frogs were cooled to -5ºC over 12 h then held for an additional 48 h before being sampled frozen(Fig. 2.1). Frozen wood frogs were sampled 6h after the initiation of ice nucleation and thawed frogs were sampled 4.5 h after thawing began at -0.6°C (Sinclair et al., 2013).

We determined glucose concentrations by homogenizing tissue samples (50 mg) with 0.6 N ice-cold perchloric acid that were then centrifuged at 2000 g for 5 minutes. Supernatant were neutralized with KOH and assayed in triplicate for glucose concentrations with a YSI-2000 analyzer and compared with a standard solution (YSI, Inc, Yellow Springs, OH, USA).

Water content was calculated as a percentage of the wet mass lost after drying. Tissues were blotted to remove excess surface moisture and weighed. Tissues were then dried at 65°C for 24 h and reweighed. Percent water was determined by subtracting post-drying weight from pre-drying weight and dividing by pre-drying weight.

Sample means (mean±SEM) were compared for significant differences using analysis of variance (ANOVA) followed by post-hoc Tukey multiple comparisons. Data of glucose concentrations were log- transformed to meet assumptions of normality and equal variance.

Statistical tests were performed in R (version 3.02) using Rstudio (version 0.98.944). Significance of statistical analyses was accepted at $P < 0.05$.

**Results**

Repeated freeze-thaw cycles in wood frogs resulted in significant step-wise increases in average glucose concentrations in all tissues (Fig. 2.2; liver: $F_{5,24} = 33.34$, $P<0.001$, heart: $F_{5,24} = 37.55$, $P<0.001$, muscle: $F_{5,24} = 30.13$, $P<0.001$). Post-hoc comparisons found no differences ($P>0.3$) in glucose concentrations in liver, heart, and leg tissues between Group 2 and Group 7 wood frogs; these groups were frozen only once but differed in how long they remained frozen before sampling. Glucose concentrations in Group 3 frogs that were frozen once and sampled after thaw remained elevated and did not differ from concentrations present in Group 2 animals sampled after freezing once ($P>0.25$). Group 4 frogs that were frozen twice and sampled frozen had significantly higher glucose concentrations in all tissues compared to levels in Groups 2 and 3 frogs ($P<0.002$ and $P<0.0001$, respectively). Glucose concentrations in Group 5 frogs, frozen twice and sampled thawed, remained elevated and did not differ from levels in Group 4 frogs ($P>0.5$). Finally, glucose concentrations were highest and differed significantly from all other groups in frogs of Group 6, which experienced three freezing events and were sampled frozen ($P<0.05$, all groups). Glucose concentrations in μmol $g^{-1}$ fresh weight (and then given in μmol $g^{-1}$ dry weight) in Group 6 frogs were 684.4 ± 144 (1477±151.5) in liver, 140.7 ± 31.2 (450.0±47.8 2) in leg muscle, and 328.9 ± 60.3 (812.5±57.82) in heart (Table 2.1, Fig. 2.2).

Water content as a percentage of fresh mass in liver, heart, and leg tissues decreased in Group 2 compared to Group 1 frogs after the initial freezing event ($F_{5,24} = 7.91$, $P<0.001$ $F_{5,24} = 13.33$, $P<0.0001$, $F_{5,24} = 3.63$, $P < 0.01$, respectively). Percent water did not differ significantly among frogs in thawed or frozen Groups 2-7 (Fig. 2.3; $P<0.05$). Tissues in Groups 2-7 all appeared

shriveled which indicated desiccation. Groups 2, 4, 6, and 7 all had coelomic ice while Groups 3 and 5 did not.

**Discussion**

In this study we demonstrate how natural daily cycling in environmental temperatures results in high glucose concentrations observed in tissues sampled from field-frozen wood frogs, despite high cooling rates (Larson et al., 2014). Our results reveal that repeated ice inoculation with short intervals of thawing causes a stepwise accumulation of glucose in wood frog tissues. Exposure to successive freezing events and the resulting high glucose concentrations may contribute to the enhanced cold tolerance observed in wood frogs overwintering under natural conditions in Alaska (Larson et al., 2014).

In our previous study of wood frogs overwintering under natural conditions, we found wood frogs from interior Alaska in natural hibernacula experience 10-17 successive freeze-thaw events before remaining frozen until spring. To protect cells from damage during and after freezing, wood frogs produce and inundate tissues with glucose and urea. Production of glucose occurs mainly in the liver with its synthesis initially stimulated by the formation of ice across the wood frog's skin (Storey and Storey, 2005). Since naturally frozen wood frogs had higher concentrations of glucose than found in previous laboratory studies, we hypothesized that the repeated freezing events observed in field animals would produce the higher concentrations observed in field-frozen wood frogs. After only three freezing events laboratory tissue concentrations approached peak concentrations observed in naturally freezing wood frogs in Interior Alaska (Fig. 2.2).

Glucose concentrations did not decrease in tissues of thawing wood frogs between freezing events (Fig. 2.2). Glucose levels may remain high due to the short thaw duration (4.5 h of thawing before sampling), a slow rate of reabsorption of glucose due to low temperatures, an inhibition of

the conversion of glucose to glycogen, or a combination of these effects. Thawed wood frogs from Ontario, Canada held at 3°C maintain elevated levels of glucose in liver, heart, and muscle tissues for several days with half-lives of 1.9, 6.2, and 8.1 days, respectively (Storey and Storey, 1986). If the half-life of glucose is similar in Alaskan wood frogs to lower latitude wood frogs, then thawing intervals observed in natural hibernacula in Alaska, which are no longer than 32 h, should not result in a significant loss of glucose from tissues between freezing episodes. Field-frozen wood frogs can experience temperatures as high as 10°C between freezing events, but the average maximum temperature during thaw periods was 3.9 ±2.8°C (Larson et al., 2014). Costanzo et al., (2014) demonstrated that captive Alaskan wood frogs held at 4°C for 5 d post-thaw still maintained high concentrations of glucose in all tissues compared to unfrozen controls. Further, post-thaw glucose concentrations decrease at a slower rate for Interior Alaskan wood frogs than Ohio conspecifics (Costanzo et al., 2014). Ohioan wood frogs also do not possess as large of stores of glycogen as their Alaskan counterparts (Costanzo et al., 2013). Wood frogs from Ohio have lower glycogen reserves after freezing despite not producing as much glucose as Alaskan (Costanzo et al., 2013). Costanzo et al., (2013) propose that high urea concentrations in wood frog blood prevent transporters from moving glucose out of cells. The cryoprotection offered by this high retention of glucose within tissues can then be augmented with further ice nucleation events that produce additional glucose.

In most laboratory studies of freeze tolerance of wood frogs, a single ice nucleation followed by a slow rate of cooling has been used to maximize glucose production and distribution and frog survival. These studies found that wood frogs frozen at high cooling rates both produce less glucose and suffer greater rates of mortality than wood frogs frozen at slower rates, usually -0.05°C $h^{-1}$, (Costanzo et al., 1992; Costanzo et al., 2013). In the field in Alaska, however, wood frogs cooled at rates of between -0.35 and -1.6°C $h^{-1}$, lethal rates for laboratory wood frogs (Costanzo et al., 1991;

Larson et al., 2014). Wood frogs frozen in the laboratory are usually cooled and held 24-48 h at the target temperature to ensure complete freezing (Costanzo et al., 2013). Wood frogs freezing at high rates in the field likely do not freeze to their final winter ice-water equilibrium during initial freezing events, since these last only 2-16 h (Larson et al., 2014). In this study only Group 7 animals were held at below freezing for a long enough to likely reach maximal ice content (Fig. 2.1). Costanzo et al., (2015) exposed wood frogs to freeze-thaw cycles by cooling wood frogs from 4 to -4°C over 160 h (6.7 days) and then warming wood frogs back to 4°C and holding for 12 h before cooling again to -4°C for 160 h. This process was repeated three times before wood frogs were cooled to and held at -8°C for 5 h. In contrast wood frogs in Group 6 underwent three freezing events in 60 h while remaining frozen for only >30 h (Fig. 2.1).

Wood frogs in this study synthesized fresh weight concentrations of glucose in the liver of 684.4 ± 144 compared which approached the concentrations found in field-collected wood frogs of 788±98.8 while freeze-thaw wood frogs from Costanzo et al., (2015) had concentrations of 437.8±113.9 (Costanzo et al., 2015; Larson et al., 2014; Fig. 2.2). Costanzo et al., (2015) wood frogs did demonstrate high concentrations of glucose in μmol $g^{-1}$ dry weight in leg muscle with 491.5±169.8 in freeze-thaw wood frogs compared with 205.9±64.4 in wood frogs cooled to -16°C at -0.05°C $h^{-1}$. Liver glucose concentrations in μmol $g^{-1}$ dry weight were higher in wood frogs cooled to -16°C with concentrations of 1312.3±351.7 while freeze-thaw wood frogs had glucose concentrations of 837.2±152.0 (Costanzo et al., 2015). In contrast, in our study Group 6 wood frogs had high glucose concentrations in both liver and leg muscle in μmol $g^{-1}$ dry weight of 1447.0±151.5 and 450.0±47.8, respectively (Table 2.1).

The lower concentrations within liver tissues from Costanzo et al. (2015) freeze-thaw experiments may be due to long thaw periods that lasted for over 6 days, which is longer than the

half-life times reported for glucose reabsorption in liver tissues (Storey and Storey, 1986). In a freeze-thaw study by Storey (1987), wood frogs from southern Ontario, Canada were frozen three times by placing animals in a temperature chamber held at -2.5°C for the first two exposures and -4°C for the final exposure. Frogs were thawed to 3°C. Each thawing and freezing period lasted 2 days. Glucose tissues concentrations in liver and lung were the only tissues out of 11 that were higher after three freezing events when compared to the first freezing event. Further, during thaw periods glucose was not retained at high concentrations, but instead appeared to be converted back into glycogen (Storey, 1987). Our animals were cooled at fast rates to mimic natural hibernacula conditions; thus, our experiments tested the impact of freeze-thaw cycles on glucose production in more ecologically relevant conditions.

Both subarctic field-frozen wood frogs and the freeze-thaw wood frogs reported on here exhibited comparable or higher glucose concentrations in all tissues relative to levels in slowly cooled wood frogs that underwent a single freezing event (Costanzo et al., 2015; Costanzo et al., 2014; Larson et al., 2014). Although wood frogs that experience slow freeze-thaw cycles have comparable concentrations of glucose in leg muscle to our freeze-thaw wood frogs, the slowly cooled freeze-thaw wood frogs exhibit lower concentrations of glucose in liver tissue than rapidly cooled freeze-thaw wood frogs (Costanzo et al., 2015; Table 2.1). Further, wood frogs cooled slowly to -16°C exhibit only slightly lower hepatic concentrations than rapidly cooled freeze-thaw wood frogs and had lower glucose concentrations in leg muscle than freeze-thaw frogs from this study (Costanzo et al., 2015; Table 2.1). Wood frogs cooled to -16°C had a higher functional concentration of glucose in the liver due to greater water loss than wood frogs in this study (Fig. 2.3; Costanzo et al., 2015). This suggests that during freeze-thaw cycles more glucose is being distributed to peripheral tissues from the liver than during a single freezing event (Costanzo et al., 2015). Our

wood frogs were able to maintain high concentrations of glucose within the liver while still distributing large quantities to peripheral tissues despite rapid cooling rates.

Tissues dehydrate during freezing and this alone can result in increases in cryoprotectant concentrations without additional glucose synthesis (Costanzo et al., 1992). Wood frogs frozen to -16°C over 320 h exhibited dehydration as high as 66% water loss in liver (Costanzo et al., 2015). This level of dehydration resulted in fresh weight glucose concentrations in liver tissues comparable to those in field-frozen wood frogs (Costanzo et al., 2015). In this study, percent water loss was as high as 46% in tissues (Fig. 2.3). Our data suggest that field-frozen wood frogs are capable of achieving high glucose tissue concentrations without high levels of dehydration (Fig. 2.3). Since water concentrations between single ice nucleation and freeze-thaw groups did not significantly differ, we believe that increases in glucose concentration were the result of additional glucose production and not further dehydration of tissues (Fig. 2.3). Further, this signifies that after initial freezing events minimal water loss is occurring from tissues within wood frogs during early freeze-thaw cycling (Fig. 2.3). The high tissue desiccation observed by Costanzo et al., (2015) could be caused by slow cooling rates wood frogs experienced.

Previous studies that examined results on cryoprotection from patterns of freeze-thaw cycling have used freeze-tolerant insects. Survival decreased in some freeze-tolerant insects exposed to multiple freeze-thaw cycles compared to when frozen once, a result that was attributed to increased energy expenditure in the insects experiencing cycles (Marshall and Sinclair, 2012). However, cryoprotection accumulation is higher in larvae of *Pyrrharctia isabella* (Smith, 1797), a freeze-tolerant Lepidoteran, after five freeze-thaw exposures compared to after one freezing event (Marshall and Sinclair, 2011). Conversely, freeze-avoiding insects, such as *Epiblema scudderiana* (Clemens, 1860) and *Belgica antarctica* (Jacobs 1900), survive at a greater rate after exposure to

repeated non-freezing, sub-zero temperatures and produce more cryoprotectants than single cold exposure insects (Churchill and Storey, 1989; Teets et al., 2011).

Other factors that may enhance freeze-tolerance in wood frogs are antifreeze glycolipids and urea. Antifreeze glycolipids, found in cell membranes, may prevent ice from propagating into the cytoplasma, stabilize cell membranes, and inhibit ice recrystallization (Walters et al., 2011). Urea is mainly accumulated prior to hibernation, although there is evidence that some urea synthesis is initiated in the liver upon freezing (Costanzo and Lee, 2005; Costanzo et al., 2015). Alaskan wood frogs display significantly higher concentrations of urea in tissues than in Ohioan wood frogs (Costanzo et al., 2013). The combination of high field glucose concentrations after freezing and high urea concentrations prior to freezing as well as the presence of antifreeze glycolipids likely all contribute to the extreme freeze tolerance of Alaskan wood frogs compared to more southerly populations (Costanzo et al., 2013; Larson et al., 2014).

Wood frogs from Alaska and Ohio frozen at a slow rate of cooling to high sub-zero temperatures display similar levels of glucose in tissues; however, Alaskan wood frogs frozen under the common laboratory method still have large stores of glycogen within their livers and hepatocytes after freezing (Costanzo et al., 2013; Costanzo et al., 2014; do Amaral et al., 2015). These large stores of glycogen indicate Alaskan wood frogs are capable of producing more glucose than southern conspecifics. Ohioan wood frogs may not have the large stores of glycogen to survive low temperatures even if exposure to multiple freeze-thaw cycles enhances cryoprotection production. Southern wood frogs may be better adapted to brief periods frozen with long periods thawed since subnivean temperatures in more southern ranges of the wood frog do not reach below 0°C for extended periods of time (Sinclair et al., 2013).

Our results demonstrate that high rates of cryoprotectant synthesis in wood frogs can be achieved through short, successive ice inoculations. Wood frogs in this study created glucose concentrations in tissues that were equivalent or higher than glucose concentrations reported in other laboratory studies despite freezing faster and being exposed to low temperatures for shorter periods. As long as thawing times remain brief wood frogs can increase tissue glucose concentrations with each successive freezing period. By using freeze-thaw cycling in laboratory experiments we can achieve similar physiological responses as seen in the field. These methods will allow for better testing of lower lethal limits for both temperature and endurance in freeze-tolerant amphibians.

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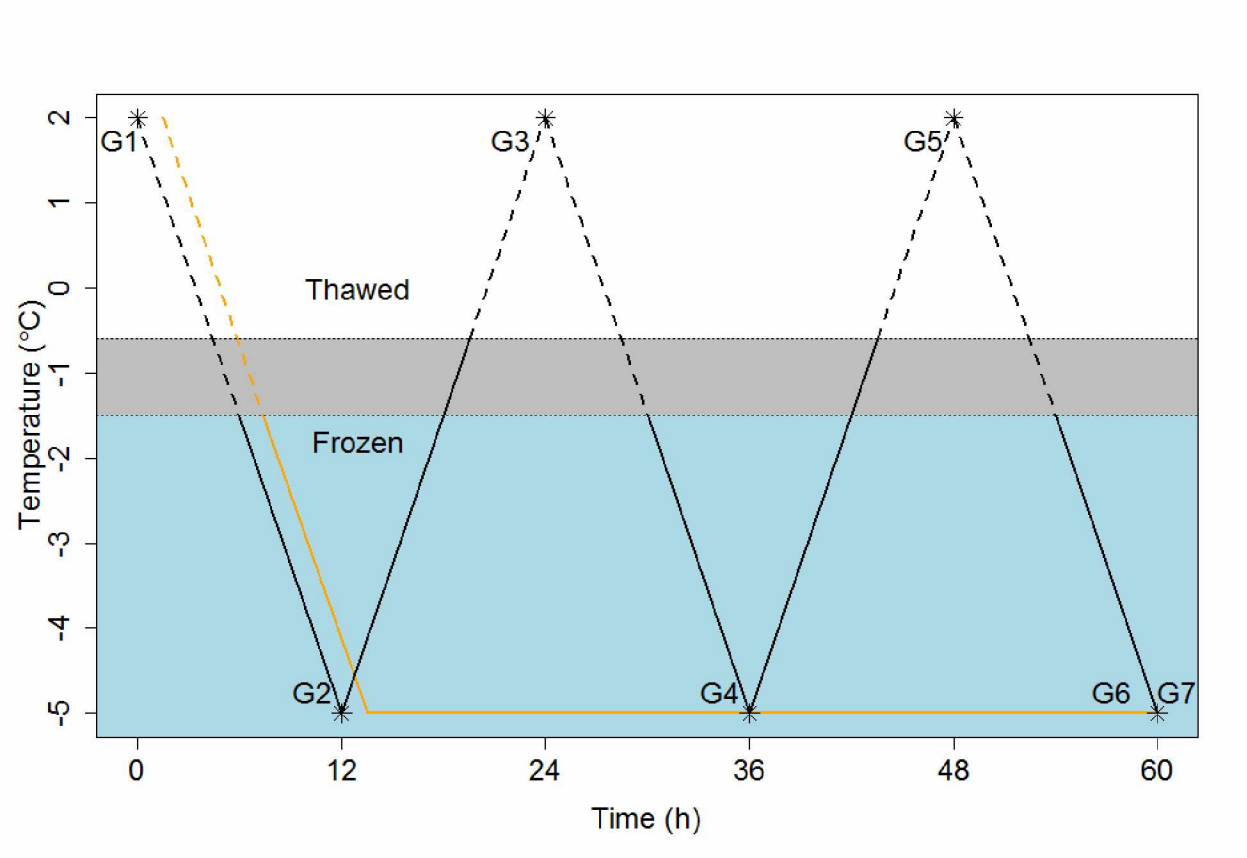


Fig. 2.1. Programmed bath temperatures over time in freeze-thaw cycles (black line) and single ice nucleation (orange line) experiments. Wood frogs were cooled or warmed at a rate of 0.58°C $h^{-1}$. Dashed lines indicate periods when wood frogs were thawed while solid lines indicate periods when wood frogs were frozen. Asterisks indicate sampling points (n=5 for each point). Blue bar indicates periods when frogs were frozen. White bar indicates thawed periods. Grey bar indicates the thawing temperature. Individuals will remain frozen until the upward bound and thawed until the lower bound of this bar. G indicates group (G1- Group 1, G2- Group 2, G3- Group 3, G4- Group 4, G5- Group 5, G6-Group 6, G7-Group 7). Lines offset for clarity.

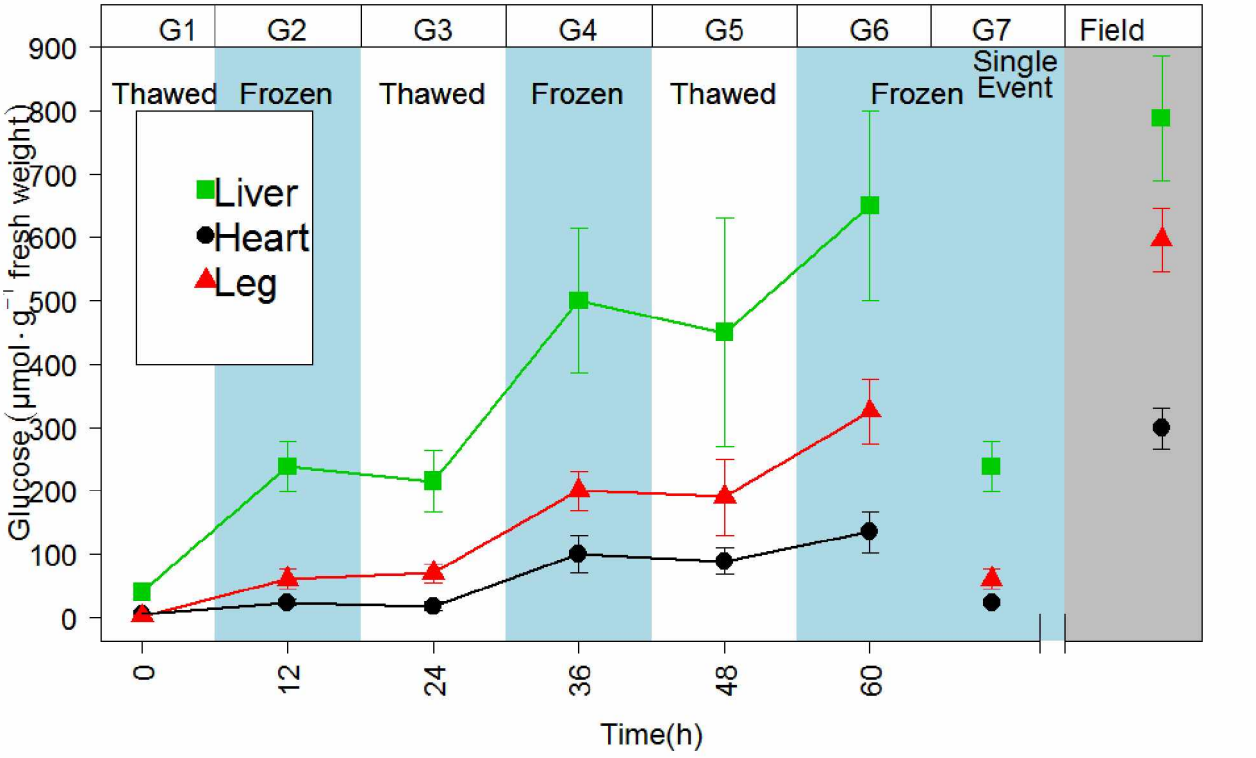


Fig. 2.2. Liver, heart, and leg muscle tissues glucose concentrations sampled from freeze-thaw cycling, single freezing event, control, and field-frozen wood frogs (Larson et al., 2014) (n=5 for G1-G7, n=34 for Field). Error bars represent ±SEM. Solid lines do not indicate temporal changes within individuals over time but trends between groups. Field-frozen wood frogs are presented for comparison. Break in x-axis indicated field-frozen wood frogs were collected in Spring after several months frozen. G indicates group. Single freezing event offset for clarity. Glucose concentrations increased with each freezing event across all tissues [liver: $F_{5,24}$= 33.34, P<0.001, heart: $F_{5,24}$= 37.55, *P*<0.001, muscle: $F_{5,24}$= 30.13, *P*<0.001]

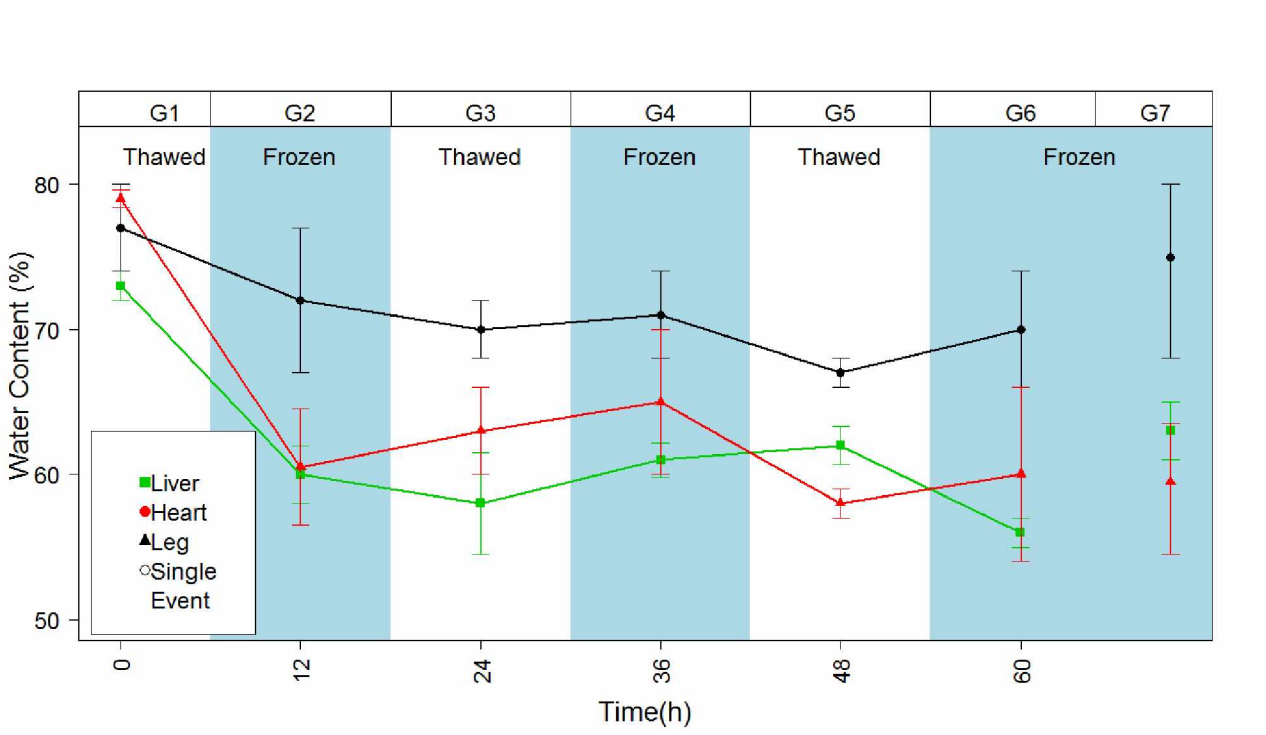


Fig. 3.3. Water content as a percentage of fresh mass from liver, heart, and leg muscle tissues sampled from control and freeze-thaw cycling wood frogs (n=5 for each point). Error bars represent ±SEM. G indicated group. Solid lines do not indicate temporal changes within individuals over time but trends between groups. G indicates group. Water content as a percentage of fresh mass differed significantly by treatment. No difference was detected between frozen and thawed groups ($P > 0.10$). Only Group 1 was significantly different from the other groups ($P<0.001$) [liver: $F_{5,24}= 7.91$, $P<0.001$, heart: $F_{5,24}= 13.33$, $P<0.0001$, muscle: $F_{5,24}= 3.63$, $P < 0.01$]

Table 2.1. Glucose concentrations (μmol $g^{-1}$ dry weight) in liver, heart, and thigh muscle in each group of wood frogs as mean±SEM (n=5 for all groups)

| Treatment | Times frozen | State at sampling | Liver | Heart | Thigh Muscle |
|---|---|---|---|---|---|
| Group 1 | 0 | Unfrozen | 35.2± 9.9 | 9.0±0.6 | 14±2.5 |
| Group 2 | 1 | Frozen | 595.5±40.2 | 153.2±15.6 | 85.0±15.6 |
| Group 3 | 1 | Thawed | 511.9±49.2 | 189.2±14.6 | 60.0±14.6 |
| Group 4 | 2 | Frozen | 1282.1±115.4 | 571.4±28.5 | 344.8±28.5 |
| Group 5 | 2 | Thawed | 1184.2±182.4 | 452.4±59.4 | 269.7± 59.4 |
| Group 6 | 3 | Frozen | 1477.0±151.5 | 812.5±57.8 | 450.0±47.8 |
| Group 7 | 1 | Frozen | 615±42.1 | 138±22.4 | 84±18.9 |

## Chapter 3 Ribeiroia ondatrae metacercariae survival in the freeze-tolerant wood frog (*Lithobates sylvaticus*)[1]


### Abstract

The larval cysts of the trematode *Ribeiroia ondatrae* were examined for survival within freeze-tolerant wood frogs (*Lithobates sylvaticus*) and whether survival was dependent on host adaptations to freezing. One hundred and seven tadpoles from Interior Alaska were exposed to 30 *R. ondatrae* cercariae each. After metamorphosis, unfrozen, control frogs (n = 22) were held for 2 wk at 2 C. Frogs experiencing a single freezing event (n = 26) were cooled from 2°C to -6°C over 12 hr, nucleated with ice to initiate freezing at -1.5°C, and then held for 2 wk at -6°C. Frogs experiencing an ecologically relevant repeated freeze-thaw (n = 29) were cooled over 12 hr from 2°C to -6°C, nucleated at -1.5°C, and then warmed over 12 hr to 2°C; this cycle was repeated twice then frogs were held at -6°C for 2 wk. Frogs (n = 10 per group) averaged 18.7 ± 2.7 motile metacercariae prior to freezing and there were no changes in abundance among unfrozen frogs after 2 wk. Freezing significantly decreased parasite survival. No parasites survived in the single freezing event group; however, parasite survival was 23% in the freeze-thaw group. Parasite survival in this group showed a positive linear relationship with the cryoprotectant (glucose) produced by the frog host. These results reveal how ecologically relevant conditions are necessary to evaluate parasite survival. This research also demonstrates how host overwintering physiology can detrimentally affect parasite survival. Additionally, these results indicate parasites may use host cryoprotectants to survive freezing.


[1] Don Larson. 2019. Ribeiroia ondatrae metacercariae survival in the freeze-tolerant wood frog (*Lithobates sylvaticus*). *Journal of Parasitology. Accepted*

## Introduction

Climate change is rapidly warming the Arctic (Trenberth et al., 2007). Warmer summers in the Arctic have not only allowed for range expansion of animal species, including non-native parasites, but have also resulted in faster development and transmission of native parasites (Kutz et al., 2005; 2013). While warmer summers have altered life history traits and ranges of parasites, winters are warming at an even greater rate (Serreze et al., 2000). How warmer winters may change life history traits and parasite ranges is unknown. To understand how climate change will alter parasitism in the Arctic, the overwintering strategies of parasites need to be better understood.

Obligate parasites at high latitudes must cope with their hosts' overwintering strategies. Hosts may overwinter by seeking thermal refugia, migrating, remaining active, or hibernating. Hibernating ectothermic hosts may be freeze tolerant or resist freezing through supercooling (cooling a liquid below its freezing point without crystallization) and/or the use of biological antifreeze (Costanzo and Lee, 2013). Endoparasites that use ectothermic hosts that experience temperatures below freezing can either avoid freezing by supercooling or tolerate freezing (Costanzo and Lee, 2013).

Previous research on helminth freeze-tolerance has focused on lethal lower temperatures related to the safety of food handling and preservation, with an emphasis on sylvatic diseases such as *Trichinella spiralis* and *Alaria* spp. (Dick and Belosevic, 1978; Chadee and Dick, 1982; González-Fuentes et al., 2015). Northern subspecies of *T. spiralis* are capable of surviving 12 mo at -15°C within carcasses of polar bears (*Ursus maritimus*) and over 20 mon frozen within Arctic fox (*Vulpes lagopus*) carcasses (Brandly and Rausch, 1950; Dick and Belosevic, 1978; Dick, 1983). Parasitoid nematodes are also capable of survival in freeze-tolerant insect hosts (Hawes and Wharton, 2011). Both free-living and parasitic nematodes have been capable of surviving below freezing

temperatures through freeze-avoidance and freeze-tolerance. Some species of nematode are even capable of surviving intracellular freezing, which is lethal for other freeze-tolerant organisms (Wharton and Ferns, 1995). Woodhams et al., (2000) examined supercooling and freeze-tolerance in *Rhabdias ranae*, a common adult nematode found in the lungs of the wood frog (*Lithobates sylvaticus* [LeConte, 1825]), and were shown to survive to -5°C both supercooled and frozen on a cryostage.

In contrast, little is known about trematode adaptations to inhabiting hosts at below freezing temperatures, although infections of larval trematodes can increase host mortality in overwintering mud snails (*Ecrobia ventrosa* and *Hydrobia acuta*) (Jensen et al., 1996). Trematodes may serially use multiple ectothermic species, such as amphibians and snails, as hosts; therefore, trematodes may experience below freezing conditions during multiple stages of their life cycle.

*Ribeiroia ondatrae* (Price, 1931) is a trematode with a 3-host life cycle where amphibians are the second intermediate host and birds such as ducks and cranes are the definitive (final) host (Beaver, 1939). Cercariae shed from planorbid (ramshorn) snails encyst as metacercariae near developing limb buds where they can induce malformations by increasing the concentration of retinoic acid (Johnson et al., 1999; Szuroczki et al., 2012). These malformations are a primary cause of amphibian declines throughout North America (Johnson and Sutherland, 2003). Although *R. ondatrae* is a common parasite in major migratory pathways and has been detected as far north as southern British Columbia, Canada (Roberts and Dickinson, 2012; Johnson et al., 2002), neither *R. ondatrae* nor parasite induced malformations have been observed in Alaska (Reeves et al., 2010; 2013; DJ Larson, pers. obs.). However, all 3 hosts of *R. ondatrae* (planorbid snails, wood frogs, and migratory birds) are present in Interior Alaska (Martoff and Humphries, 1959; Maciolek, 1989; Armstrong, 2008).

The wood frog ranges from the Appalachians to north of the Arctic Circle (Martoffand Humphries, 1959). In the northern region of this range wood frogs overwinter in small depressions under snow and leaf litter (Kirton, 1974; Larson et al., 2014). Overwintering wood frogs can experience temperatures below 0°C for only a few days at low latitudes and up to 7 mo at high latitudes (Sinclair et al., 2013; O'Connor and Rittenhouse, 2015; Groff et al., 2016; Larson et al., 2014). Within hibernacula Alaskan wood frogs survive average winter temperatures of -6.3°C and average minimum temperatures of-14.6 ± 2.8°C (range -8.9 to -18.1 C) (Larson et al., 2014).

By producing cryoprotectants, urea and glucose, wood frogs can tolerate extracellular freezing (Storey and Storey, 1985; Costanzo and Lee, 2005; Larson et al., 2014). As ice forms in the extracellular fluid osmotic gradients draw water from cells. By increasing solute concentrations of glucose and urea within cells, wood frogs can tolerate cellular desiccation due to freezing (Storey and Storey, 1988; Costanzo and Lee, 2005). Urea, glucose, and antifreeze glycolipids also prevent intracellular freezing, stabilize membranes, and prevent the lethal propagation of ice across cell membranes (Storey and Storey, 1988; Duman, 2015). Urea is synthesized in high concentrations prior to freezing; however, glucose is only synthesized and distributed systemically in response to ice inoculation across the skin (Storey and Storey, 1988; Costanzo and Lee, 2005). Wood frogs frozen in natural hibernacula can produce concentrations of over 900 μmol $g^{-1}$ fresh weight of glucose within tissues. Maximum cryoprotectant concentrations measured in frogs collected in the field can be replicated in the lab by exposing frogs to repeated freeze-thaw cycles that mimic natural conditions (Larson and Barnes, 2016).

Some parasites have demonstrated the ability to use host produced antifreeze proteins to survive freezing conditions (Kolb, 2013); therefore, parasites may also be able to use host cryoprotectants to survive freezing. Low temperature conditions experienced by hosts during

Alaskan winters may limit *R. ondatrae*'s northern range. I hypothesize that parasite survival will be dependent on host cryoprotectant production and freezing will lower the mean intensity of infection compared to unfrozen wood frogs. Here *R. ondatrae* were tested for survival within a freeze-tolerant host during freezing and if that survival is dependent on host physiology.

**Materials and Methods**

One hundred and seven wood frog tadpoles (Gosner stage >25) were collected by hand in the Fairbanks North Star Borough (64.8°N, 147.8°W) during July and August 2011 (Gosner, 1960). Tadpoles were held in 38 L aquaria (n = 10 per aquarium) at 21°C with a 8L/16D light cycle, which are comparable conditions to local ponds (Herreid and Kinney, 1967). Water was changed weekly. Tadpoles were fed daily 0.5 g Tetra Spirulina Enhanced fish flakes (if this can be purchased anywhere then you do not need to supply the name of the manufacturer, city, state in the USA; name, city country outside the USA, but if not then you do need to supply this information). Once all tadpoles reached Gosner stage 32 or greater, each was exposed to 30 *R. ondatrae* cercariae. To prevent malformations tadpoles were only exposed to cercariae after limb bud development had begun. Cercariae were obtained from infected snails collected from Boulder, Colorado by holding snails in individual 50 ml vials in the dark for 12 hr and isolating cercariae using a pipette while viewing through a stereomicroscope. Thirty cercariae were then pipetted into 50 ml vials and added to 100 mL containers containing individual tadpoles. After 48 hr tadpoles were then placed back in their original aquarium. When tadpoles reached Gosner stage 42, an embankment of rocks was placed in aquaria to allow metamorphosing wood frogs to climb out of the water.

Metamorphosed wood frogs (n = 10 per aquaria) were transferred to 38 L aquaria with moss and rock with water available in a 10 ml dish. The temperature of the aquaria was decreased to 5°C and held there for an additional 2 wk. Wood frogs were divided into 3 groups: unfrozen control,

single freezing event, and freeze-thaw, n = 32, 36, and 39, respectively. Ten wood frogs were euthanized from each group with an overdose of MS-222 and necropsied to determine mean number of pre-experimentation motile metacercariae. *Ribeiroia ondatrae* metacercariae were examined with a wet mount for motility within cysts. If no movement was observed after 30 sec, metacercariae were excysted (removed from their cyst) by applying light pressure to the coverslip with a pair of fine forceps.

For freeze-tolerance experiments, the remaining wood frogs were placed in individual 200 ml containers on top of moist moss. The control group was held unfrozen at 2°C for the remainder of the experiment. For freezing experiments, a 30-gauge type T thermocouple was attached to a recorder (Iso-Thermex, Columbus Instruments, Columbus, Ohio) against each wood frog's ventrum to measure body temperature. Containers were placed in an ethanol-water bath (Neslab ULT-80, Waltham, Massachusetts) with an initial temperature of 1 C; ice formation was initiated at -1.5°C by placing finely crushed ice on to the dorsum of wood frogs. Wood frogs were assumed thawed at -0.6°C (Sinclair et al., 2013). The single freeze group was cooled from 1 to -6°C over 12 hr (-0.58°C $hr^{-1}$) and then held for 2 wk at -6 C. The freeze-thaw cycles were similar to Larson et al., (2014) (Fig. 3.1). Wood frogs were cooled from 1 to -6°C at -0.58°C $hr^{-1}$ and then warmed them back to 1°C at +0.58°C $hr^{-1}$. The freeze-thaw cycling was repeated once more before cooling again to -6°C at -0.58°C $hr^{-1}$ and held the wood frogs for 2 wk at -6 C. Wood frogs in the freeze-thaw group therefore experienced an additional 29 hr below freezing before being held at -6°C for 2 wk (Fig. 3.1). After 2 wk, all frogs were euthanized with an overdose of MS-222, necropsied and metacercariae examined for motility as described above. Liver, heart, and gracilis major muscle tissues were collected to determine glucose concentrations.

Glucose concentrations in wood frogs were determined by homogenizing tissue samples (50 mg) with 0.6 N ice-cold perchloric acid and centrifuged at 2,000 *g* for 5 min. Extracts were neutralized with KOH and assayed in triplicate for glucose concentrations with a YSI-2000 analyzer and compared with a standard solution (YSI, Inc, Yellow Springs, Ohio).

Mean motile parasites and glucose concentrations (mean ± SEM) were compared for significant differences using analysis of variance (ANOVA) followed by post-hoc Tukey multiple comparisons. Parasite survival as a function of leg tissue glucose concentration was tested with a linear regression. Glucose concentrations were log transformed to meet assumptions of normality and equal variance. Statistical tests were performed in R (version 3.02) using Rstudio (version 0.98.944). Results were considered significant at $P < 0.05$.

Taxonomic names, concepts, and identification keys were from Beaver (1939) and Dodd (2013).

**Results**

Mean number of motile parasites isolated from wood frogs at experiment–end depended on freezing treatment (Fig. 3.2; $F_{5,101} = 61.6$, $P < 0.001$). There were no significant differences in motile parasites among groups prior to freezing ($P > 0.1$); all 3 groups averaged 18.7 ± 2.7 motile parasites per frog. The control group had no significant decrease in number of motile metacercariae after 2 wk unfrozen. No parasites isolated from wood frogs exhibited motility following the single freezing event treatment. There were significantly fewer motile parasites in wood frogs experiencing freeze-thaw cycles, 5.14 ± 3.30, compared to motile parasites in unfrozen wood frogs 17.86 ± 2.47 ($F_{5,101}=61.6$, $P < 0.001$, Fig. 3.2).

Glucose concentrations in wood frog tissues were highest in the freeze-thaw group compared to the single freeze and unfrozen wood frog groups (Fig. 3; liver: $F_{2,74} = 25.93$, $P < 0.001$,

heart: $F_{2,74} = 19.25$, $P < 0.001$, muscle: $F_{2,74} = 37.32$, $P < 0.001$). Glucose concentrations (μmol $g^{-1}$ fresh weight) in freeze-thaw wood frogs were 770.2 ± 171.2 in liver, 155.9 ± 42.6 in leg muscle, and 325.8 ± 82.3 in heart tissues compared with levels in single freeze event wood frogs that were 260.3 ± 54.6 in liver, 24.8 ± 11.8 in leg muscle, and 58.7 ± 16.5 in heart tissues (Fig. 3.3). Body size was not a covariate of muscle glucose concentration.

Within the freeze-thaw group, the number of motile parasites found in frozen wood frogs increased linearly with glucose tissue concentrations measured within frog gracilis major (Fig. 3.4) ($F_{1,27} = 28.7$, $P > 0.0001$, adjusted $r^2 = 0.4973$). The 95% confidence interval for the slope was 0.033-0.074.

**Discussion**

In highly seasonal environments such as the Arctic and Subarctic, parasites using multiple hosts may not be able to complete their life cycles in 1 summer season and therefore will spend at least 1 winter in an immature stage (Wharton, 1999; Kutz et al., 2005). During winter, survival of these parasites is dependent on their hosts' response to winter. In ectotherms this means surviving winter within a host whose body temperature can be well below freezing. Ectotherms cope with body temperatures below freezing by either avoiding freezing through supercooling, using antifreeze proteins to prevent the growth of ice, or by tolerating extracellular ice formation and preventing cellular harm through the production of cryoprotectants (Storey and Storey, 1988). This study supports my hypothesis that host physiology influences parasite survival. In this study I demonstrate how freezing in wood frogs lowers the incidence of parasitism by *R. ondatrae*, and also provides evidence that *R. ondatrae* use host cryoprotectants to increase survival during freezing.

Wood frogs in the field undergo multiple freeze-thaw cycles before remaining frozen for the rest of winter. Glucose concentrations in tissues that approached field levels were induced by

mimicking this pattern of natural field freeze-thaw cycles in the lab (Larson and Barnes, 2016). Wood frogs frozen with freeze-thaw cycling had higher concentrations of glucose than wood frogs frozen with 1 freezing event (Fig. 3.3). This allowed a comparison of the effect of low and high glucose concentrations on survival of metacercariae.

Parasites in unfrozen wood frog larvae did not decline in motility over 2 wk (Fig. 3.2). This result supports field observations of Bolek and Coggins (2001) where helminth communities within green frogs (*Lithobates clamitans*) did not significantly differ between October and April. Green frogs overwinter in ponds and lakes and remain unfrozen during winter (Lamoureux et al., 1999). After infected wood frog metamorphs were frozen by a single freezing event and held for 2 wk at -2 C, no motile parasites were collected, indicating that freezing in this circumstance was lethal to encysted metacercariae (Fig. 3.2). Woodhams et al., (2000) froze parasitized wood frogs for 4 days under similar conditions to the single freezing group. They reported observing motility in Echinostome metacercariae collected from frozen wood frog kidneys, although the authors did not include data on the percent of viable motile metacercariae. In the present experiment only 23% of metacercariae frozen in the freeze-thaw group were motile post freezing (Fig. 3.2). The survival of Echinostome metacercariae observed by Woodhams et al., (2000) could be attributed to higher concentrations of glucose found in kidneys than in leg muscle as well as the brief periods of subzero temperatures hosts were exposed to.

Even though the percent of motile parasites decreased with freezing, in the freeze-thaw group the number of motile parasites recovered among individuals increased with host leg muscle glucose concentrations (Fig. 3.4). Muscle is the best proxy available for cryoprotectant concentrations that metacercariae would experience as all *R. ondatrae* found were encysted in the inguinal region or tail resorption site. *Clinostomum marginatum* metacercariae have both active and

passive glucose transportation across the metacercariae cyst wall (Uglem and Larson, 1987; Larson et al., 1988). Like *R. ondatrae* metacercariae, *C. marginaturm* encyst within muscle and connective tissue (Hopkins, 1933). *R. ondatrae* metacercariae are most likely increasing their survival by passively absorbing high concentrations of glucose. Glucose concentrations available were likely too low following a single freezing event to provide metacercariae with protection against extracellular ice formation. Urea is an additional important cryoprotectant in wood frogs but is unlikely to contribute to freeze-tolerance in metacercariae as trematodes cannot transport urea into metacercariae and thus would only draw water out of metacercariae (Barret, 2000). Unlike glucose, urea concentrations are increased prior to, not during, freezing (Costanzo and Lee, 2005). The increased osmotic pressure that urea exerts on parasites would not be significantly different in unfrozen and frozen hosts. Urea may increase osmotic stress on parasites prior to or during winter causing a decrease in parasite survival. Since the number of motile parasites present was strongly correlated with glucose concentrations in nearby tissues, *R. ondatrae* survival may be enhanced by maximal wood frog glucose (cryoprotectant) accumulation. The highest parasite survival would be expected in habitats where wood frogs do not freeze during winter.

Low survival of parasites in freezing hosts may reduce parasitism enough to prevent the establishment of *R. ondatrae* and other parasites in wood frogs overwintering in Alaska. Parasite diversity in wood frogs in Alaska is low. Surveys of wood frog parasites in Kenai National Wildlife Refuge, Alaska, USA from 2004-2010 found only *Echinostoma* spp. metacercariae (Reeves et al., 2013). Surveys of wood frogs in the Fairbanks North Star Borough have found *Rhabdias* spp. and *Echinostoma* spp. (D. Larson pers. obs.). *Alaria* spp. has been observed in Alaska from definitive hosts such as wolves (*Canis lupus*), red fox (*Vulpes vulpes*), and wolverines (*Gulo gulo)* (Babero and Rausch, 1952; Rausch, 1959; Rausch and Williamson, 1959). *Alaria* spp. can use wood frogs as an

intermediate host but has not yet been documented in Alaskan wood frogs. *Alaria* spp., like *Echinostoma* spp. and *Rhabdias* spp. may also be freeze-tolerant and should be examined for winter survival in wood frogs (Woodhams et al., 2000). Freezing may contribute to the low parasite diversity currently observed in Alaska wood frogs.

Much like some nematode species in the Arctic and Subarctic, trematodes of wood frogs may require two or more years to complete their life cycles (Kutz et al., 2005). Freezing across multiple winters may lower parasite mean intensity (average infection per infected host) within wood frog hosts to prevent successful completion of trematode life cycles, thereby resulting in the lack of parasitism and parasite-induced malformations in Interior Alaska. Parasites may instead overwinter within other common trematode hosts like snails or definitive hosts such as birds or mammals. Snails at high latitudes may overwinter within frozen lakes and ponds by avoiding freezing, although their overwintering behavior is not well documented (Oswood et al., 1991).

Summer conditions may also be a barrier to the establishment of *R. ondatrae* in the Arctic and Subarctic. *Ribeiroia ondatrae* eggs will not develop in water below 12°C (Paull and Johnson, 2011). Temperatures in Interior Alaska ponds and lakes can remain below this threshold temperature well into July (Herreid and Kinney, 1967). If egg development does occur, cercariae may not develop fast enough within snails to infect tadpoles that leave natal ponds in late July to early August. Wood frog tadpoles metamorphose between 53 and 78 days after hatch (Herreid and Kinney, 1967). Infected snails will begin releasing cercariae by 50 days at 20 C; thus, development would take longer in Interior Alaskan ponds where summer temperatures average 17.4°C (Herreid and Kinney, 1967; Paull and Johnson, 2011). Wood frog tadpoles may not be present in ponds and lakes when cercariae are being released.

If overwintering within wood frogs is necessary to complete the life cycle of *R. ondatrae* then these results suggest that winter may lower but not prevent the introduction of *R. ondatrae* into Alaska. Current projections for warmer winters in the Arctic and Subarctic indicate that *R. ondatrae* could become established in the future if freezing is a major barrier to range expansion. At lower latitudes wood frogs produce less glucose and remain frozen for fewer days (Costanzo et al., 2013; Sinclair et al., 2013; Groff et al., 2016). Even if parasite survival is dependent on glucose production, parasites may still have high survival when frozen for short durations since longer term freezing may be lethal (Woodhams et al., 2000). Although climate change may result in warmer winters, it can also result in changes to precipitation. Since wood frogs depend on the snow pack for insulation, any decrease in snow accumulation will result in lower temperatures in the subnivean space (Pauli et al., 2013). Lower temperatures may also result in reduced parasite survival since only 23% survived at -6°C (Fig. 3.4). Not only will climate change disrupt winter hibernacula, but it may also result in phenological mismatches among hosts such as earlier shedding of cercariae in snails due to earlier spring thaw or higher temperatures within overwintering ponds (Paull and Johnson, 2011).

Freeze-tolerance is associated with high survival in freeze-tolerant amphibians; however, the same cannot be said for helminth endoparasites of hibernators (Larson et al., 2014; Berman et al., 2016). Changes in host physiology due to winter influence parasitism rates. Survival for these parasite communities is dependent on the physiological responses hosts have to winter and the parasites' ability to cope with these changes. This study demonstrates that increased cryoprotectant production by hosts can increase parasite survival (Fig. 3.4). Both host and parasite overwintering strategies need to be considered when predicting changes in parasite ranges and life histories at high latitudes.

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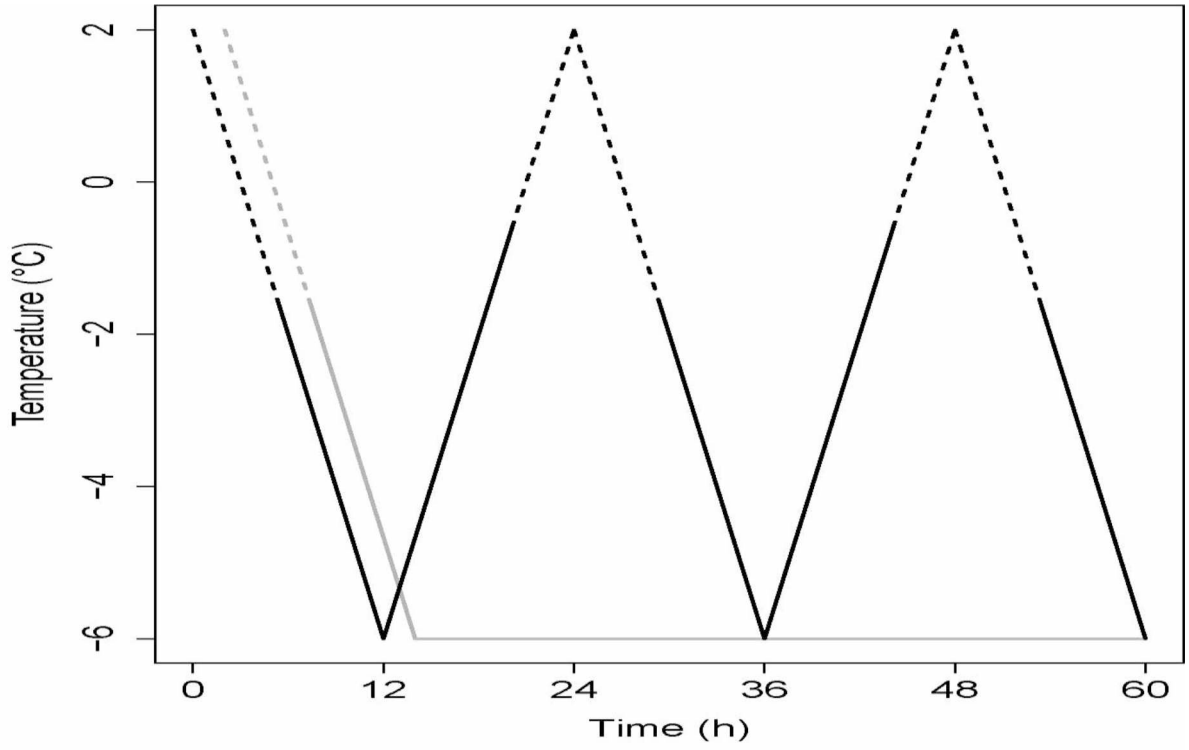


Figure 3.1. Programmed bath temperatures over time in freeze-thaw cycles (black line) and single ice nucleation (gray line) experiments. Dashed lines indicate periods where wood frogs were thawed while solid lines indicate periods where wood frogs were frozen. Lines offset for clarity.

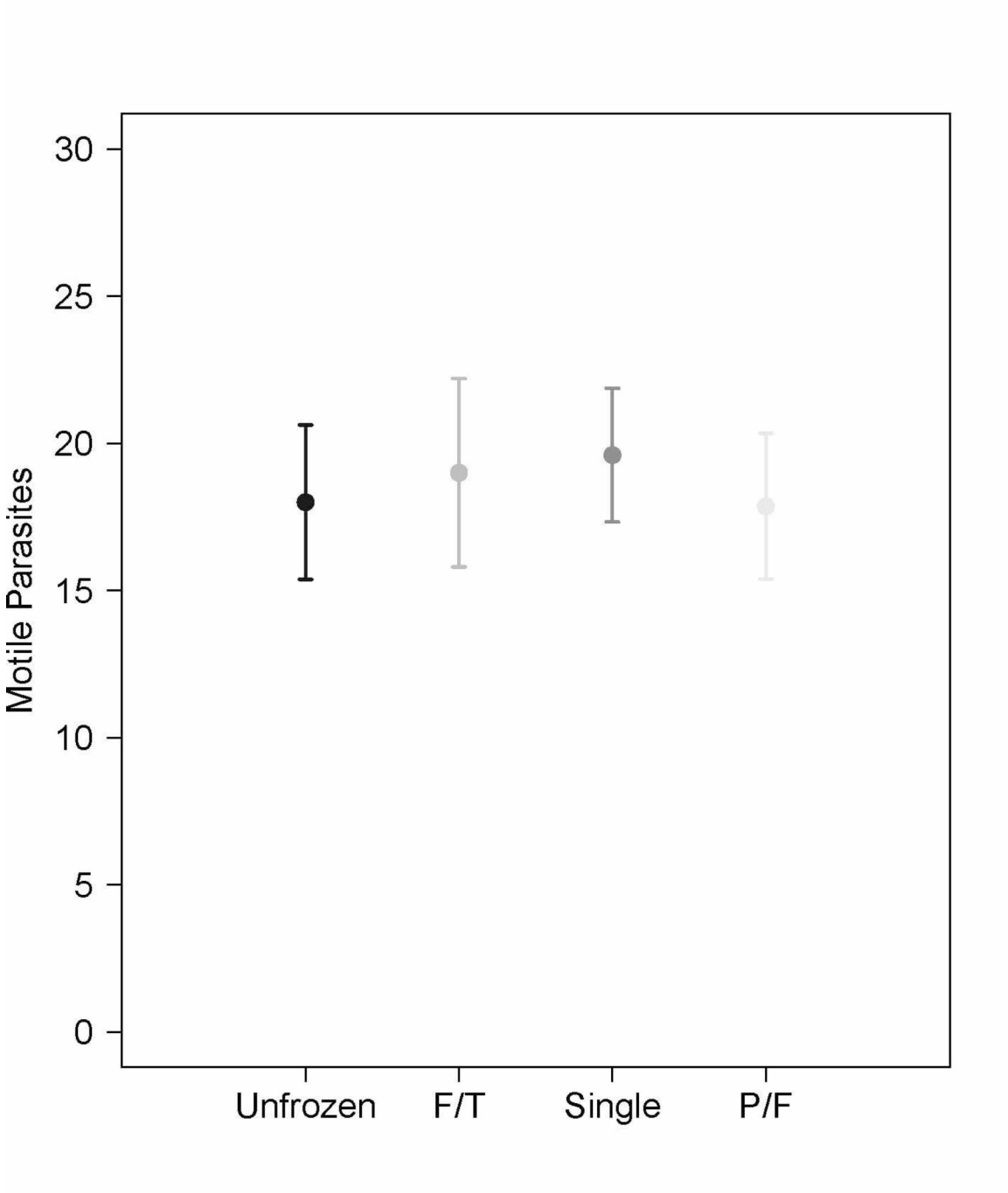


Figure 3.2. Motile parasites (Mean±SEM) observed in unfrozen controls, freeze-thaw cycles (F/T), single freezing event (Single), and prior to experimentation (P/FO treatments prior to freezing exposure (n=22, 26, 29, and 30, respectively). Parasite motility differed significantly post freezing among all groups ($F_{5,101} = 61.6$, $P < 0.001$)

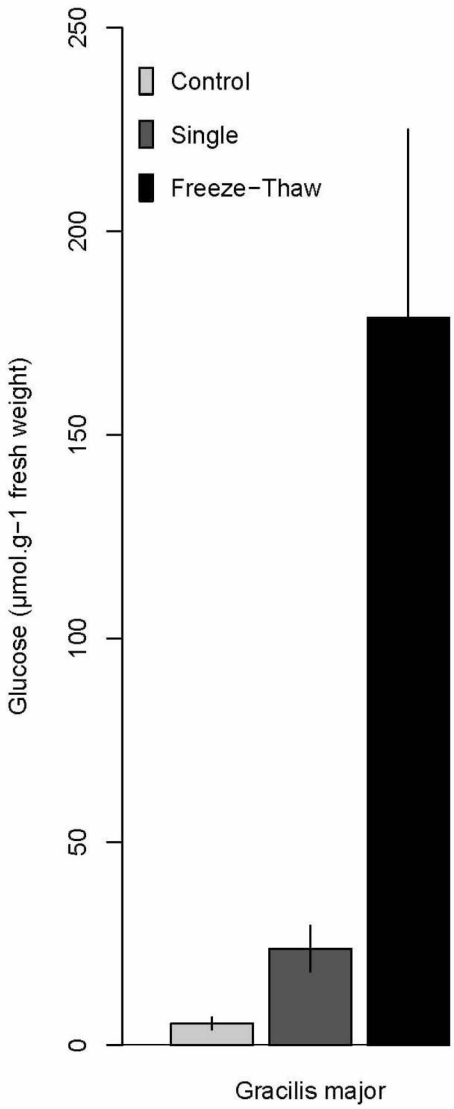


Figure 3.3. Gracilis major tissue glucose concentrations sampled from freeze-thaw cycling, single freezing event, unfrozen wood frogs (n=10, for each group). Error bars represent ±SEM. Glucose concentrations differed significantly [$F_{2,74} = 37.32$, $P < 0.001$]

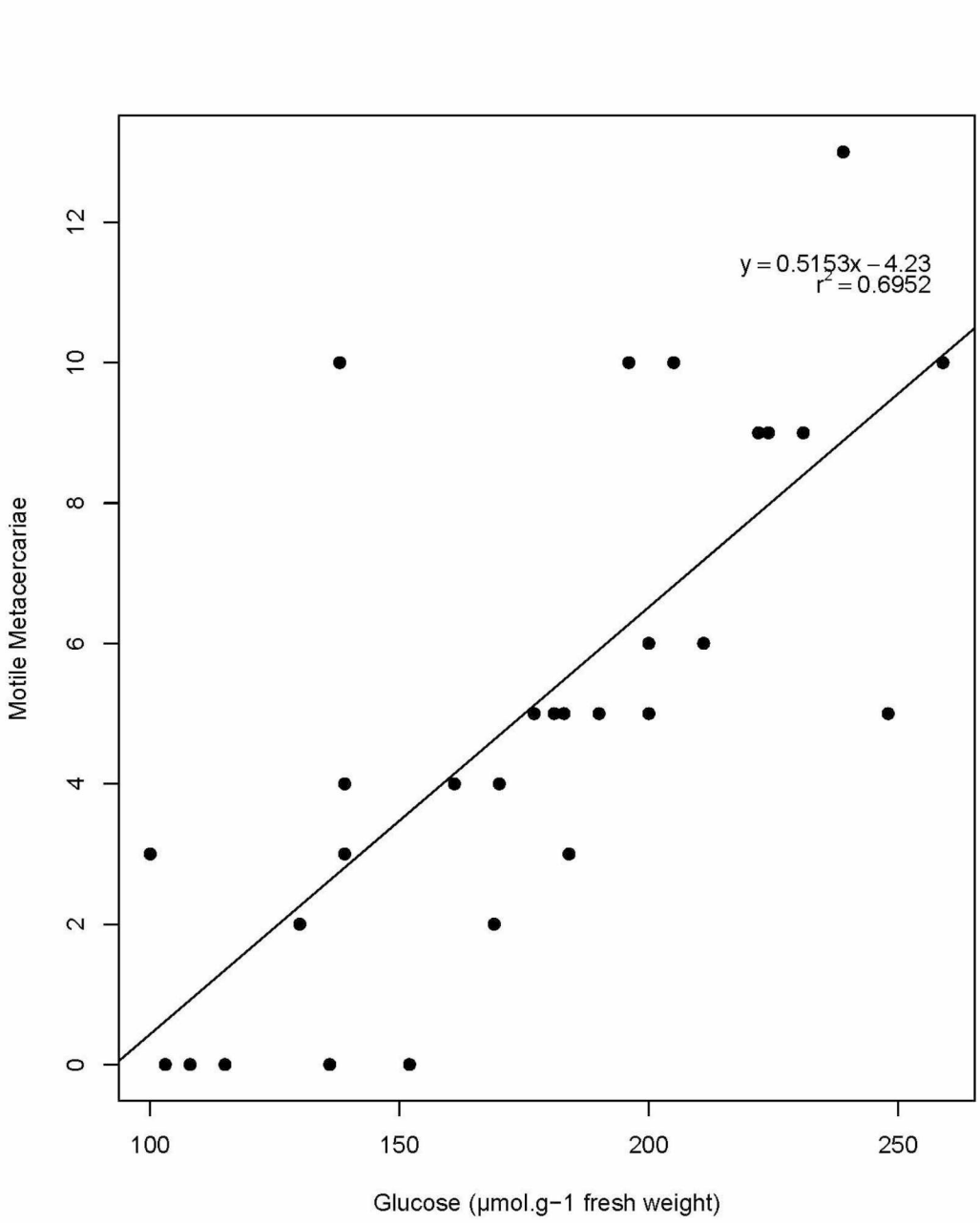


Figure 3.4. Motile cercariae as a function of gracilis major glucose concentrations (µmol $g^{-1}$ fresh weight) in freeze-thaw metamorphs (n = 29) ($F_{1,27}$=28.7, $P < 0.0001$, adjusted $r^2 = 0.4973$). CI 95%: 0.033-0.074.

## General Conclusion

How hosts overwinter and their physiological response to winter dictates how parasites overwinter. For parasites, a host's internal environment is its own ecosystem with patterns of seasonality in temperature, food availability, immune activity, and other physiological processes that are in turn a response to the seasonality of a host's external environment. Parasite overwintering survival is predicated on host winter physiology and parasite adaptability to these changes. In order to understand how a warming Arctic will change host-parasite interactions and parasite communities more research is needed on parasite overwintering strategies. Future work should treat hosts overwintering physiology as a changing environment for parasites and examine the implications of host overwintering strategies on parasite survival instead of parasites as only a detriment host winter survival. Examining host winter physiology and parasite survival will be key to predicting range expansions of non-native parasites and changes in parasite abundance in the Arctic.

My work studied the delicate winter relationship between parasites and hosts by using wood frogs and *R. ondatrae* as a model. I demonstrate that host overwintering physiology can both inhibit and assist parasites. Future work on host-parasite overwintering needs to balance an understanding of parasitology with ecophysiology. Combining these fields allows for a broad approach to answer future questions on not only where do parasites go during winter but how climate change will impact both parasite and host populations.

My thesis follows a clear path of understanding host physiology in order to ask questions about parasite overwintering. In order to understand host-parasite interactions during winter and how host physiology influences these interactions, I had to understand the environment of *R. ondatrae* during winter. This meant determining the natural conditions wood frogs experience during winter and understanding how to mimic these conditions in a laboratory setting. This first chapter

set new limits both the endurance and low temperature threshold for wood frogs overwintering. Wood frogs were able to survive up to 7 months frozen with temperatures as low as -18°C. My work is also the first to describe overwintering hibernacula for wood frogs as well as demonstrating that wood frogs in the field produce higher concentrations of glucose than previously reported. I hypothesized from this chapter that the daily temperature cycling causing freeze-thaw cycles in hibernacula allowed for progressive increases of glucose with each subsequent freezing event. My second chapter supported this hypothesis and demonstrated that multiple freeze-thaw cycles could result in the high glucose concentrations seen in field wood frogs. These two chapters allowed me to design an experiment to test the effect of cryoprotectant production on parasite survival. By using a more natural freeze-thaw cycle than a long, slow cool I was able to mimic glucose concentrations in the field. After analyzing the data, it became clear that glucose concentrations strongly correlated with parasite survival. Had I only tested parasite survival using the commonly accepted practice of freezing wood frogs at -0.05°C I may not have observed the correlation between glucose concentration and parasite survival or even parasite survival. This result emphasizes the importance of testing parasite overwinter with sound ecophysiological methods that consider field conditions.

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**Institutional Animal Care and Use Committee**
909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

August 26, 2011

| | |
|---|---|
| To: | Brian Barnes, PhD<br>Principal Investigator |
| From: | University of Alaska Fairbanks IACUC |
| Re: | [259022-3] Wood Frog freeze tolerance under natural conditions |

The IACUC reviewed and approved the Amendment/Modification referenced below by Designated Member Review.

| | |
|---|---|
| Received: | August 26, 2011 |
| Approval Date: | August 26, 2011 |
| Initial Approval Date: | August 26, 2011 |
| Expiration Date: | August 26, 2012 |

This action is included on the September 13, 2011 IACUC Agenda.

*The PI is responsible for acquiring and maintaining all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol, and could result in revocation of IACUC approval.*

University of Alaska Fairbanks

## *INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE*

John E. Blake DVM MVSc
Chair and Attending Veterinarian
Institutional Care and Use Committee
University of Alaska Fairbanks
PO Box 757000, 311 Irving I Building
Fairbanks, Alaska 99775-7000

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June 7, 2001

**Subject: IACUC review of *Assurance of Animal Care* form**

Dear Dr Barnes

The following revised protocol using vertebrates was reviewed by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) at their June meeting. This protocol was approved by the following vote:

5 FOR and 0 AGAINST (3 Absent)
WITH MODIFICATIONS (see below). Minority opinions, if any, are indicated below.

| | |
|---|---|
| IACUC Protocol Number: | 01-07 |
| Investigator/Instructor: | Dr. Barnes |
| Title of Project/Course: | Wood Frog (*Rana sylvatica*) Overwintering Behavior and Physiology |
| Date Received: | 2/23/01 (revision received 3/14/01) |
| Date Approved: | 6/7/01 (permission to acquire frogs given in April) |
| Annual Renewal: | June (annual report is due) |

*This protocol will be valid for **12 months after approval** and must be kept current, especially with respect to new methods or techniques as they evolve. Please see the Appendix at the end of*

# University of Alaska Fairbanks

## *INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE*

### INSTRUCTIONS REGARDING AN APPROVED *ASSURANCE OF ANIMAL CARE* FORM

As the principal investigator or course instructor, you will receive a letter indicating the date and outcome of the vote for the final review of the *Assurance of Animal Care* form (*Assurance*). Attached to this letter will be the most current (and active) version of your *Assurance*. Since it may contain changes from your original submission you MUST carefully review the form and any stipulations or requirements set by the committee. Major modifications or requirements set by the IACUC have been incorporated into your *Assurance* and are listed at the end of your approval letter. If you are asked for a response you MUST do so within 14 days of the date on the letter or your *Assurance* will be suspended. Minor changes may not be listed on the letter but have been incorporated into the form, therefore, you should read the *Assurance*! If you feel changes have been made that will interfere with your research objectives please notify the committee immediately; otherwise you are to conduct your research or class using the most current version of the form. The *Assurance* must be kept current by submitting modifications when needed, especially with respect to new methods or techniques as they evolve. Check the Appendix at the end of the form listing significant changes to animal use protocols that require submission of a written modification to the IACUC. Deviation from the activities outlined in your current *Assurance* is NOT permitted unless you have received approval from the IACUC for modifications. Conduct of unapproved activities will necessitate suspension of ALL your animal research activities and notification of your funding agency(ies).

*Access to your Assurance:*

You are required to maintain a readily accessible copy of your *Assurance* in your laboratory and/or office and you must ensure that all personnel working on this project read and understand the protocol.

*Life Span of the Assurance and Reporting Requirements:*

All *Assurances* are valid for 12 months after approval and must be kept current with respect to new methods or techniques as they evolve. You will receive notification from the IACUC during the month prior to the annual anniversary of your *Assurance*. In this notice you will be asked if your project is finished or if it is continuing. Additionally, you will be given instructions for filing your annual report on the previous year's activities conducted under this protocol. At that time you may renew the protocol for another 12-month period. It may be renewed for a maximum of two times. Thereafter, a new *Assurance* must be filed with the IACUC. NOTE: an annual report is required whether you renew the *Assurance* or not!

*Formal Training Requirement for Personnel Working with Live Vertebrates:*

All personnel working with live vertebrates must complete the appropriate module(s) of the University's web-based training program in animal care and use. All individuals performing manipulations on vertebrates (handling, capture, blood collection, surgery, etc.) must demonstrate proper training, experience, and capability. It is the principal investigator's responsibility to ensure that all individuals working on their protocol have received adequate training. It is the Institution's responsibility to ensure that proper training is made available. Contact the UAF attending veterinarian for information about our training programs.

*Notifying Funding Agencies about IACUC Protocol Review:*

You must notify the IACUC Recording Secretary if a letter confirming IACUC review and approval is required by a granting agency. We can issue a formal letter for you; however, it is your responsibility to ensure this is done and to provide the required contact and agency information prior to the grant deadline.

*Additional Information:*

Visit the IACUC web site or contact any member of the IACUC.

*the form listing significant changes to animal use protocols to determine whether a written modification must be submitted. As stipulated in the Animal Welfare Act and Public Health Service Policy, this protocol may be renewed annually by the PI for a maximum of 2 renewals. One month prior to each anniversary of your Assurance you will receive a review form from the IACUC. On the third anniversary of this Assurance you will be notified of its termination. At that time, if the project is continuing, you will need to submit a new protocol for review. All lab animals and captive wildlife used under this Assurance of Animal Care Form* ***must*** *be identified with the assigned IACUC number by using cage cards, door cards, or some ready method of identifying pens or paddocks with this Assurance.*

As a condition of approval, the IACUC requires the following modifications or clarification to the above referenced application. Most, if not all, have already been incorporated into the *Assurance*. Items in RED require your response within 14 days of the date on this letter or the protocol will not receive final approval.

1) Surgeries to implant the telemetry devices into the body cavity must be done under veterinary supervision until the veterinarian determines that the surgeon is ready to work unsupervised.

2) The committee emphasizes the importance of maintaining permanent quarantine and adherence to disease containment procedures to ensure that no pathogens are transmitted from Ohio or Indiana frogs to indigenous frogs.

3) The committee stipulates twice daily observations on frogs to ensure that disease problems are quickly identified and that veterinary services is contacted for a timely necropsy.

4) Housing of frogs in room 269 (Barne's lab) makes this area a satellite facility. It will be inspected every six months by the IACUC during the AHRB animal facility inspection for as long as you are holding frogs.

NOTE: Federal regulations and guidelines governing the care and use of animals in research and teaching require that all individuals working with live vertebrates are now required to complete a formal training program. To accommodate busy schedules, this is being developed as a web based course at UAF. During 2001, you and your staff will be asked to complete the appropriate sections of this course so the IACUC has formal documentation of your knowledge of animal welfare.

Minority Opinion: none

John Blake
Chair, IACUC

Attachments: approved *Assurance of Animal Care* form
Instructions regarding an approved *ASSURANCE OF ANIMAL CARE* form

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**Institutional Animal Care and Use Committee**

909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

August 26, 2011

To: Brian Barnes, PhD
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [259022-3] Wood Frog freeze tolerance under natural conditions

The IACUC reviewed and approved the Amendment/Modification referenced below by Designated Member Review.

| | |
|---|---|
| Received: | August 26, 2011 |
| Approval Date: | August 26, 2011 |
| Initial Approval Date: | August 26, 2011 |
| Expiration Date: | August 26, 2012 |

This action is included on the September 13, 2011 IACUC Agenda.

*The PI is responsible for acquiring and maintaining all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol, and could result in revocation of IACUC approval.*

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**Institutional Animal Care and Use Committee**
909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

May 17, 2011

To: Brian Barnes, PhD
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [241531-1] Parasite Freeze Tolerance

The IACUC reviewed and approved the New Project referenced below by Full Committee Review.

| | |
|---|---|
| Received: | May 6, 2011 |
| Approval Date: | May 16, 2011 |
| Initial Approval Date: | May 16, 2011 |
| Expiration Date: | May 16, 2012 |

Note: If DoD funding is to be used to support this project, the DoD ACURO will need to review the project before activities can begin.

This action is included on the May 12, 2011 IACUC Agenda.

*The PI is responsible for acquiring and maintaining all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol, and could result in revocation of IACUC approval.*

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**Institutional Animal Care and Use Committee**
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May 23, 2011

To: Brian Barnes, PhD
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [241528-2] Tadpole Capture and Transport SOP

The IACUC reviewed and approved the Amendment/Modification referenced below by Designated Member Review.

| | |
|---|---|
| Received: | May 20, 2011 |
| Approval Date: | May 23, 2011 |
| Initial Approval Date: | May 23, 2011 |
| Expiration Date: | May 23, 2012 |

This action is included on the May 23, 2011 IACUC Agenda.

*The PI is responsible for acquiring and maintaining all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol, and could result in revocation of IACUC approval.*